Functional studies on the histidine kinase CaNik1p from *Candida albicans*

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English Summary

There is a continuous need to evaluate new drug targets which allow the development of antimicrobial therapies with minimal side effects in humans. Histidine kinases (HKs) are essential components of two-component signal transduction (TCST) systems that are absent in mammalian cells. Thus, they are considered drug target candidates. CaNik1p is a group III HK of the most frequent human fungal pathogen *Candida albicans*. The relevance of this group of enzymes for fungicidal activity was shown by the construction of *Saccharomyces cerevisiae* transformants, in which CaNik1p and other group III HKs were expressed. These transformants were sensitive to antifungals, to which wild-type *S. cerevisiae* was resistant. However, the mode of action of the fungicides is still not completely understood.

To investigate the role of different domains of CaNik1p for fungicidal activity, wild-type and mutated variants of *CaNIK1* were constructed and heterologously expressed in *S. cerevisiae*. Although group III HKs are known to be relevant for certain classes of antifungals, it was not clear whether they are direct or indirect targets for these antifungals. Via Saturation Transfer Difference-NMR we could show for the first time a direct interaction between fludioxonil and CaNik1p, an example of group III HKs. The *S. cerevisiae* strains, which expressed variants of the protein, which were mutated in the conserved HisKA, HATPase_c and REC domains, showed complete loss of sensitivity against the tested antifungals. The sensitivity to antifungals correlated with the phosphorylation state of the MAP kinase Hog1p after treatment with fludioxonil. However, fludioxonil did not interfere with the histidine kinase activity of the protein as revealed from *in vitro* kinase assays using the purified protein and monitoring ATP consumption or autophosphorylation of the protein. The kinase activity could even be detected in all of the mutated variants. Group III HKs are characterized by consecutive HAMP domains in the N-terminal part of the protein. As the function of these domains is largely unknown, we deleted them from the protein. Expression of CaNik1pΔHAMP led to growth inhibition of the transformed *S. cerevisiae* that correlated with constitutive phosphorylation of Hog1p. This could be reversed by additional point mutation in the conserved His510 of the HisKA domain or by expression in *S. cerevisiae* with single gene deletion mutants of the response regulator *SSK1* or one of the components of the Hog1 MAPK module. The expression of the mutated variants of *CaNIK1* in *C. albicans*, however, was not yet successful.

In conclusion, treatment with antifungals and deletion of HAMP domains led to severe growth inhibition, which correlated with the activation of the MAP kinase Hog1p, and for which the functionalities of the conserved domains of the HK CaNik1p were essential.

German Summary (Zusammenfassung)

Histidinkinasen (HK) sind essentielle Bestandteile von Zweikomponenten – Signaltransduktionssystemen, die es in menschlichen Zellen nicht gibt. Daher werden sie als mögliche Angriffspunkte für Wirkstoffe betrachtet, die antimikrobielle Therapien mit minimalen Nebenwirkungen im Menschen ermöglichen. CaNik1p ist eine Histidinkinase (HK) der Gruppe III aus dem humanpathogenen Pilz *Candida albicans.* Die Relevanz dieser Enzymgruppe für die Wirkung von Fungiziden wurde durch die Herstellung von *Saccharomyces cerevisiae* – Transformanten gezeigt, in denen CaNik1p und andere Gruppe III HKs exprimiert wurden. Diese Transformanten waren für verschiedene antimykotische Verbindungen empfindlich, für die der Wildstamm der *S. cerevisiae* resistent war. Die genauen Wirkmechanismen dieser Fungizide sind jedoch noch nicht verstanden.

Um die Rolle von verschiedenen Domänen von CaNik1p für die fungizide Wirkung zu untersuchen, wurden Punktmutationen in den konservierten Domänen HisKA, HATPase_c und REC eingeführt und diese mutierten Proteine und das Wildtyp-Protein in *S. cerevisiae* heterolog exprimiert. Obwohl die Relevanz der Gruppe III HK für Fungizide bekannt ist, war die direkte Wechselwirkung noch nicht gezeigt worden. Mit dem rekombinant hergestellten, aufgereinigten Protein CaNik1p konnten wir diese Wechselwirkung mit dem Wirkstoff Fludioxonil über Sättigungstransferdifferenz-NMR erstmals zeigen. Außerdem erwiesen sich die Transformanten, die die mutierten Proteine exprimierten, als resistent gegen die Fungizide. Die Sensitivität gegenüber Fungiziden korrelierte mit dem Phosphorylierungszustand der MAP kinase Hog1p nach Behandlung mit Fludioxonil. Fludioxonil inhibierte jedoch die Kinaseaktivität des Proteins nicht, wie wir durch In-vitro Kinaseaktivitätstests mit dem gereinigten Protein zeigen konnten. Die Kinaseaktivität konnte selbst in den Proteinmutanten noch nachgewiesen werden. Gruppe III HK sind durch eine Abfolge von HAMP Domänen im N-terminalen Bereich der Proteine charakterisiert. Da die Funktion dieser Domänen noch weitgehend unverstanden ist, entfernten wir sie aus dem Protein. Die Expression dieses verkürzten Proteins CaNik1pΔHAMP führte zur Wachstumshemmung der *S. cerevisiae*-Transformante, was mit der konstitutiven Phosphorylierung von Hog1 korrelierte. Durch zusätzliche Punktmutation in der HisKA-Domäne (H510Q) oder durch Expression in *S. cerevisiae* Mutanten, in denen einzelne Gene des HOG-Signalweges deletiert waren, konnte dieser Effekt aufgehoben werden. Die Expression von mutierten Varianten von *CaNIK* in *C. albicans* war noch nicht erfolgreich.

Die Behandlung mit Fungiziden und die Deletion der HAMP Domänen führten zur Reduktion des Wachstums, für die die Funktionalität der konservierten Proteindomänen essentiell war.

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List of Abbreviations

5-FOA	5-Fluoroorotic Acid
˚C	Degree Celsius
µ	Micro, 10^{-6}
Ω	Ohm
%	Percent
A	Alanine
A	Absorbance
aa	Amino acid
Ab	Antibody
ACN	Acetonitrile
ADP	**A**denosine **di****p**hosphate
Ala	Alanine
APS	**A**mmonium **p**er**s**ulfate
Asn	Asparagine
Asp	Aspartic acid
ATP	**A**denosine **t**ri**p**hosphate
BCA	**B**i**c**inchoninic **a**cid
BISA	**Bi**ological **S**ystems **A**nalysis
BLAST	**B**asic **l**ocal **a**lignment **s**earch **t**ool
bp	**B**ase **p**air
BRENDA	**Br**aunschweig **En**zyme **Da**tabase
BSA	**B**ovine **s**erum **a**lbumin
C. albicans	*Candia albicans*
Ca	*Candia albicans*
Ca.	Circa
Cat. No.	Catalog number
CCD	**C**harge-**C**oupled **D**evice
Ci	Curie
D	Aspartic acid
Da	Dalton

DMSO	**D**i**m**ethyl **s**ulf**o**xide
DNA	**D**eoxyribo**n**ucleic **a**cid
DTT	**D**i**t**hio**t**hreitol
E. coli	*Escherichia coli*
e.g.	For example
E1%	Mass Extinction Coefficient (L. gm-1cm-1) for a 10 mg/ml of a substance (1 %)
EDTA	**E**thylene**d**iamine**t**etraacetic **a**cid
et al.	And others
Fig.	Figure
FLAG	A proprietary synthetic epitope tag consisting of eight amino acids (Asp–Tyr–Lys–Asp–Asp–Asp–Asp–Lys) and readily detected by using anti-FLAG antibodies.
FLP recombinase	A yeast enzyme that catalyses site-specific recombination of double-stranded DNA at a 34-nt sequence called the FLP recombinase target (FRT) site.
FRT	Specific 34-nt sequence recognized by FLP recombinase
G	Glycine
g	Gram
Gln	Glutamine
Gly	Glycine
GMP	**G**uanosine **m**ono**p**hosphate
H	Histidine
h	Hour
HAMP	Evolutionary conserved amino acid sequences called HAMP domains that frequently exist in **h**istidine kinases, **a**denylcyclases, **m**ethyl-accepting chemotaxis proteins, and **p**hosphatases
HATPase_c	A protein domain that is found in several ATP-binding proteins, e.g., histidine kinase, DNA gyraseB, topoisomerases heat shock protein HSP90.
His	Histidine
HisKA	His kinase A (phosphoacceptor) domain
HK	**H**istidine **k**inase
HOG	**H**igh **o**smolarity **g**lycerol

HPT	**H**istidine **p**hospho**t**ransfer protein
HRP	**H**orse**r**adish **p**eroxidase
HZI	**H**elmholtz-**Z**entrum für **I**nfektionsforschung
IC50	Concentration of compound that inhibits the growth of tested organism by 50 %
IgG	Immunoglobulin G
IMH3	**I**nosine **m**onophosphate **d**ehydrogenase
K	Kilo, 10^3
K	Lysine
LB	**L**uria-**B**ertani medium
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
M	Molar
m	Meter or Milli, 10^{-3}
mA	Milliampere
mAb	**M**onoclonal **a**nti**b**ody
MALDI-TOF MS	**M**atrix-**a**ssisted **l**aser **d**esorption/**i**onisation-**t**ime **o**f **f**light **m**ass **s**pectrometry
MAP	**M**itogen **a**ctivated **p**rotein
MAPK	**M**itogen **a**ctivated **p**rotein **k**inase
MBq	Megabecquerel
MCS	Multiple **c**loning **s**ite
mg	Milligram
MHz	Mega**h**ert**z**
min	Minute
ml	Milliliter
MPA	**M**yco**p**henolic **a**cid
MS	**M**ass **s**pectrometry
MW	**M**olecular **w**eight
MWCO	**M**olecular **w**eight **c**ut **o**ff
N	Asparagine
NMR	**N**uclear **m**agnetic **r**esonance
NST	**N**our**s**eo**t**hricin
OD	**O**ptical **d**ensity

ORF	**O**pen **r**eading **f**rame
pAb	**P**olyclonal **a**nti**b**ody
PAGE	**P**olyacrylamide **g**el **e**lectrophoresis
PBS	**P**hosphate-**b**uffered **s**aline
PCR	**P**olymerase **c**hain **r**eaction
PEG	**P**oly**e**thylene **g**lycol
PTFE	**P**oly**t**etra**f**luoro**e**thylene
PVDF	**P**oly**v**inyli**d**ene **f**luoride
Q	Glutamine
REC	**Rec**eiver domain
RP	**R**eversed **p**hase
rpm	**R**otation **p**er **m**inute
RR	**R**esponse **r**egulator protein
RT	**R**oom **t**emperature
S	Serine
S. cerevisiae	*Saccharomyces cerevisiae*
SAP2	**S**ecreted **a**spartyl **p**roteinase family 2
Sc	*Saccharomyces cerevisiae*
SDS	**S**odium **d**odecyl **s**ulfate
SD-ura	Synthetic medium containing dextrose as a carbon source and deficient in uracil
sec	Second
Ser	Serine
SG-ura	Synthetic medium containing glucose and raffinose as carbon sources and deficient in uracil
STD-NMR	**S**aturation **t**ransfer **d**ifference-**n**uclear **m**agnetic **r**esonance
T	Threonine
Tab.	Table
TAE	**T**ris-**a**cetate-**E**DTA
TBS-T	**T**ris-**b**uffered **s**aline with **T**ween-20
TCST	**T**wo **c**omponent **s**ignal **t**ransduction
TE	**T**ris-**E**DTA
TEMED	N,N,N′,N′-**te**tra**m**ethyl**e**thylene**d**iamine

TFA	**T**ri**f**luoroacetic **a**cid
TGS	**T**ris-**g**lycine-**S**DS
Thr	Threonine
Tyr	Tyrosine
Ura	Uracil
UV	**U**ltra**v**iolet radiation
V	Volt
Y	Tyrosine
YPD	**Y**east extract, **p**eptone, **d**extrose medium
YPM	**Y**east extract, **p**eptone, **m**altose medium

1 Introduction

1.1 The pathogenic fungus *Candida albicans*

Fungi affect human life in many different ways. They are useful for humans in various aspects, e.g., the production of antibiotics and alcoholic beverages, food processing (bread and cheese), and being used as food (mushrooms). On the other hand, they can cause human diseases, either directly or through their toxins, including mycotoxins and mushroom poisons (1). Among all the fungi, only a few species are pathogenic for humans. The most frequently diagnosed fungal infections are caused by pathogens from the genera *Candida*, *Cryptococcus*, and *Aspergillus* (2).

The genus *Candida* belongs to the phylum Ascomycota, class Ascomycetes, order Saccharomycetales (3). *Candida* species occur abundantly in the gastrointestinal tract and vagina. At least 17 species of *Candida* are reported to cause human disease (4-5). Infection with such opportunistic fungi has become a major concern in the caring of immunocompromised patients. *Candida* spp. is the fourth most important cause of hospital-acquired bloodstream infections, and up to 90 % of HIV patients have caught the mucosal form of the pathogen at least once (6). Among the various pathogenic species of *Candida,* e.g., *C. albicans, C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, *C. albicans* is the most frequent pathogen in over 90 % of all invasive infections (5, 7).

First described in 1839, *C. albicans* is a commensal fungus that usually exists as a benign member of the skin and mucosal flora, particularly within the gastrointestinal tract of humans and other warm-blooded animals (8).

C. albicans is an opportunistic pathogen that can proliferate even in healthy people to cause circumscribed infections of the skin, nails, and mucous membranes (8). Vulvovaginal candidiasis is common and probably affects up to 75 % of women at least once in their lifetime (9). In patients with deficient immune systems because of inherited disease, malignancy, concurrent infection, or medical intervention, this commensal yeast can behave as an aggressive pathogen, attacking virtually any organ system, and leading to death in as many as 50 % of cases of bloodstream infections (8).

C. albicans is polymorphic organism that is able to grow in three different morphological forms: as a unicellular budding yeast or as filamentous pseudohyphal and hyphal forms (Fig. 1.1) (10). This morphological plasticity of *C. albicans* is considered as a major virulence factor (11).

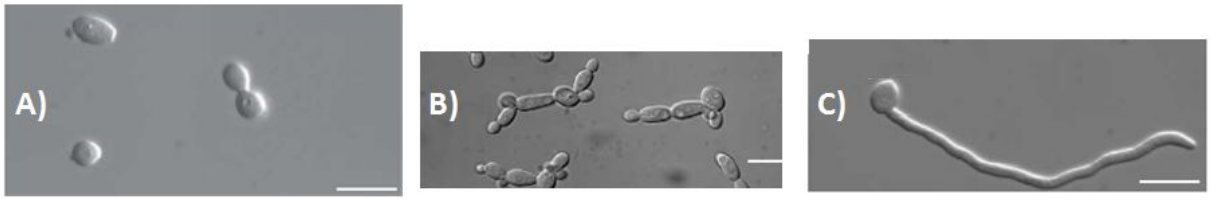


Fig. 1.1: Morphological forms of *C. albicans*. A) Yeast B) Pseudohypha C) Hypha. All scale bars represent 10 µm (10).

1.2 Antifungal agents

1.2.1 Classification

According to their mechanism of action, antifungals are mainly classified as inhibitors of fungal sterols (targeting cell membrane), inhibitors of beta-glucan synthase (targeting cell wall), or inhibitors of nucleic acid synthesis (12).

Antifungals targeting the cell membrane include polyenes, azoles, and allylamines. The polyenes (e.g., Amphotericin B and Nystatin) function by binding strongly to ergosterol to create drug-lipid complexes that intercalate into the fungal cell membrane to form a membrane-spanning channel. This causes cellular ions, such as potassium ions, to leak out of the cell, thereby destroying the membrane potential (13). The azoles (e.g., fluconazole) act by targeting the ergosterol biosynthetic enzyme lanosterol demethylase (also referred to as cytochrome P450) leading to severe membrane stress on the cell (13). Allylamine antifungals (e.g., terbinafine) are also ergosterol biosynthesis pathway inhibitors, but they inhibit earlier steps of the ergosterol biosynthesis pathway than azole derivatives, namely squalene epoxidation (13).

The echinocandins (e.g., caspofungin and micafungin) are large lipopeptide molecules that act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase, an enzyme involved in fungal cell wall synthesis. The disruption of the (1,3)-β-D-glucan network results in the loss of cell wall integrity and severe cell wall stress (13).

Flucytosine is a fluorinated pyrimidine that interferes with pyrimidine metabolism and thus with RNA/DNA and protein synthesis (12).

1.2.2 Problems with currently used antifungals

Despite the existence of several antifungals in the market, toxicity and/or the development of resistance represent major concerns (14). The toxicity of amphotericin B results from the poor ability of the drug to differentiate between ergosterol from fungal cell membranes and

cholesterol from mammalian cells (12). Moreover, amphotericin B and triazoles have been found to be associated with hepatotoxicity (15). Because a glucan-rich cell wall is a target not found in mammalian cells, echinocandins were originally predicted to be effective antifungal agents with little collateral toxicity in mammalian cells. However, they lack activity against some common opportunistic yeasts (*Cryptococcus* species) and some less common molds that usually develop as breakthrough infections in severely immunocompromised patients (15).

In addition to the toxicity associated with the use of several antifungals, the development of resistance has also been reported during the frequent administration of antifungals. Gene mutations can induce resistance, and the development of a secondary multidrug (echinocandin-azole)-resistant yeast strain has been described (16). Therefore, a great need exists to develop new antifungals with lower toxicity to humans.

1.3 Histidine kinases and the two component system

1.3.1 Kinases

Kinases are enzymes that catalyze the transfer of a phosphate group, usually from ATP, to an acceptor molecule (17). A protein kinase catalyzes the phosphorylation of protein substrates. Kinases are also known as phosphotransferases (17). They constitute a key class of enzymes responsible for the regulation of many biological phenomena (18). Kinases are involved in almost every signal transduction pathway occurring in a living cell. Although various types of biological molecules can be phosphorylated (proteins, nucleotides, sugars, lipids, etc), the largest group of kinases is that of the protein kinases, which phosphorylate in eukaryotes mainly at Ser/Thr, or Tyr residues and also at His residue (18). When the phosphorylation of a protein kinase is catalyzed by another molecule of the same kinase, the phosphorylation is called autophosphorylation (17).

1.3.2 Histidine kinases as part of the two component system in prokaryotes and lower eukaryotes

The presence of phosphohistidine in proteins was reported as early as the 1960s (19). However, the important roles of histidine phosphorylation in cellular signal transduction were not recognized until the 1980s, when “two component” systems were first identified in various bacterial signaling pathways (20-21). The prototypical two component system is composed of a histidine kinase (HK) protein, called the “sensor kinase”, and a response regulator (RR) protein (Fig. 1.2A) (19). The sensor kinase often is a transmembrane protein that detects extracellular stimuli and responds by modulating the activity of its cytoplasmic

histidine kinase domain (HisKA). When activated, the sensor kinase autophosphorylates a conserved histidine residue in the HisKA domain, after the binding of ATP to the ATP-binding domain (HATPase_c). The phosphoryl group is then transferred to a conserved aspartate residue in the receiver domain (REC) of a RR protein, resulting in the alteration of RR activity. Many RRs have a DNA-binding domain and function as transcription factors whose activity is regulated by aspartate phosphorylation in the receiver domain. Other RRs, however, can interact directly with other proteins (22). Thus, extracellular signals are transmitted by His-Asp phosphotransfer in two component systems. This mechanism of signal transduction is ubiquitous among diverse prokaryotic signaling pathways that regulate chemotaxis, sporulation, metabolite fixation, virulence, cell division, and responses to environmental stress (19).

In eukaryotes, a multistep phosphorelay (Fig. 1.2B) seems to be more prevalent than the simple two component format found in prokaryotes. All HKs in yeasts are hybrid HKs that contain both a histidine kinase domain (HisKA) and a receiver domain (REC) within a single polypeptide chain. In these enzymes, a phosphate group is first transferred from a conserved histidine residue in the kinase domain to an aspartate residue in the receiver domain. Subsequent phosphotransfer to an aspartate residue in the cognate RR is mediated by a third protein, the histidine phosphotransfer protein (HPT), that carries a histidine-containing phosphotransfer domain, in which a phosphohistidine intermediate is formed. Thus, signals are transmitted by a three step His-Asp-His-Asp phosphotransfer in phosphorelay signaling systems (19).

An essential reaction of HKs is its dimerization independently of whether it is a hybrid (22). HK autophosphorylation occurs through a trans mechanism between two molecules in the dimer. ATP bound to the ATP-binding domain of one molecule in the dimer is used to phosphorylate the histidine residue of a kinase domain of the other molecule (22).

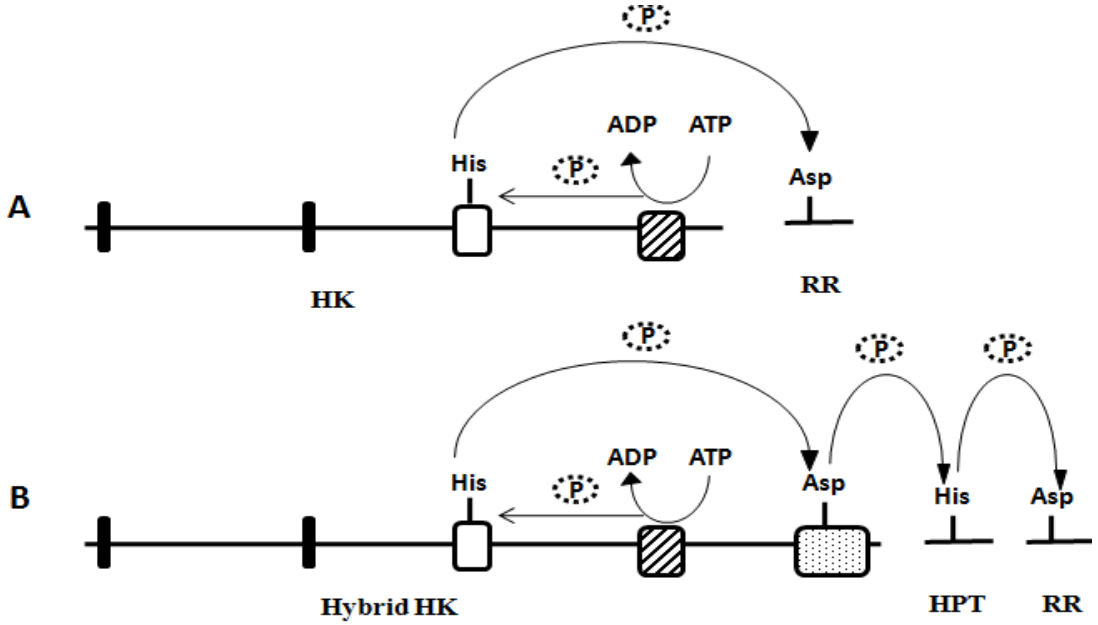


Fig. 1.2: Elements of a typical two component signal transduction system. A) Simple two component system that employs a histidine kinase (HK) and a response regulator (RR) B) Multi-step phosphorelay that employs a hybrid HK with both histidine kinase and receiver domains, a histidine-containing phosphotransfer protein (HPT), and a response regulator (RR). Regions covered by consensus domains (HisKA (□), HATPase_c (▨), REC (▩), and transmembrane domains (black bars)) are indicated.

1.3.3 Differences between prokaryotic and eukaryotic two component signal transduction systems

Several features distinguish eukaryotic two component signal transduction (TCST) systems from those of prokaryotes.

The number of TCST systems differs greatly in bacteria and fungi. In *Pseudomonas aeruginosa*, more than 130 TCST systems have been revealed by genome sequence analysis (23), whereas in *Escherichia coli*, about 30 HKs and 32 RRs have been reported (24). In contrast to the high number of TCST systems identified in prokaryotes, only a limited number have been found in eukaryotes. In the genome of the budding yeast *S. cerevisiae*, only one phosphorelay system (SLN1-YPD1-SSK1, SKN7) has been found. The pathogenic yeast *C. albicans* contains three HKs, namely CaSln1p, CaNik1p, and Chk1p (25). However, filamentous fungi generally have a far greater number of hybrid HKs than yeast-type fungi. *Neurospora crassa*, *Aspergillus nidulans*, and *Aspergillus fumigates* have 11, 15, and 14 hybrid sensor HKs, respectively (26).

Hybrid HKs make up less than 20 % of HKs in bacteria, whereas more than 90 % of eukaryotic HKs are hybrid HKs (27).

Prokaryotic RRs are predominantly transcription factors (at least 25 of the 32 in *E. coli*), whereas only one eukaryotic RR with a DNA-binding domain, Skn7p, is known in *S. cerevisiae* and *C. albicans* (28).

HPT proteins act as a key connector in the fungal phosphorelay system by relaying the phosphate group from the Asp residue of the REC domain of the hybrid sensor kinase to the Asp residue of a RR. However, in all fungi that have been studied, a single HPT protein has been identified in their genomes (26). In bacteria, HPT proteins are rare, e.g., SpoB phosphotransferase in the bacterium *Bacillus subtilis*, which is involved in the regulation of sporulation induction (29).

Many bacterial HKs (e.g., EnvZ) are able to phosphorylate and dephosphorylate (phosphatase activity) the cognate RR, e.g., the EnvZ/OmpR osmoregulation system in *E. coli* (22). To date, none of the HKs in fungi has been established as having phosphatase activity.

1.3.4 The high osmolarity glycerol (HOG) pathway as a model of fungal two component signal transduction systems

The best understood TCST system in fungi is the SLN1-YPD1-SSK1 module in *S. cerevisiae*; this module regulates the activity of the downstream mitogen activated protein kinase (MAPK) module, namely Hog1 (Fig. 1.3). In the absence of osmotic stress, transmembrane HK ScSln1p is active, so that the RR Ssk1 is phosphorylated via the HPT protein Ypd1p (30-31). Under these conditions, Ssk1p cannot activate the MAP3K Ssk2p so that the MAPK Hog1p remains inactive. Under conditions of high osmolarity, ScSln1p is inactive, and the resulting accumulation of the unphosphorylated RR Ssk1p activates the downstream MAPK module so that the MAPK Hog1p is phosphorylated (30-31). Phosphorylated Hog1p upregulates the transcription of genes encoding enzymes that play a key role in glycerol production in order to maintain the intracellular water balance, thus allowing growth under high-osmolarity conditions (26). Likewise, the disruption of *ScSLN1* results in the accumulation of unphosphorylated Ssk1p and, thus, the constitutive activation of the HOG cascade, which is lethal (30).

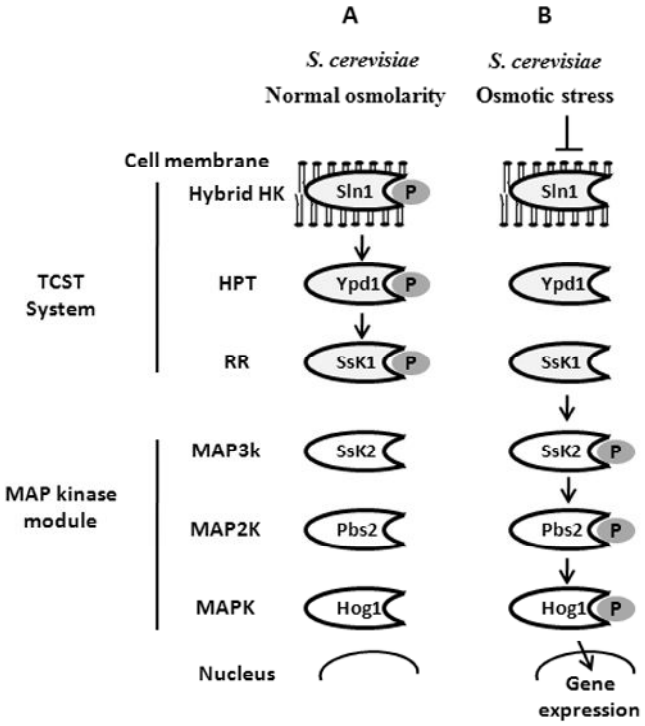


Fig. 1.3: Regulation of the Hog1 MAPK module by the TCST system SLN1-YPD1-SSK1 in *S. cerevisiae.* A) under normal osmolarity B) in the presence of osmotic stress

1.3.5 Relevance of studying two component signal transduction systems

TCSTs are involved in the adaptation of bacteria, lower eukaryotes, and plants to various environmental signals. Genome analysis has shown that such systems do not exist in *Caenorhabditis elegans*, *Drosophila*, mouse, and humans (22). The apparent absence of phosphorelay systems in the human genome might allow the development of antibiotics that specifically target TCST systems or phosphorelay proteins in pathogenic bacteria and fungi (19, 26, 32). Mammals do indeed contain some HK-like proteins, such as the Nm23 metastasis suppressor, the histone H4 HK, and the G protein β-subunit kinase, although sensor-type HKs have not been reported (26, 33).

1.3.6 Classification of the eukaryotic histidine kinases

According to the classification of Catlett *et al.* 2003, the putative fungal HKs fall into 11 classes (34). All eukaryotic HKs have HisKA, HATPase_c, and REC domains as conserved domains, whereas each class is characterized by additional characteristic domains. For example, HKs of group VI contain transmembrane domains such as ScSln1p and CaSln1p in *S. cerevisiae* and *C. albicans*, respectively. Group III HKs are characterized by additional amino acid repeats in the N-terminal region with a length of approximately 90 amino acids

each. The repeats contain evolutionary conserved amino acid sequences called HAMP domains. This abbreviation is attributable to the frequent occurrence of such domains in **H**istidine kinases, **A**denylcyclases, **M**ethyl accepting chemotaxis proteins, and **P**hosphatases, which are proteins associated with signal transduction in both prokaryotic and lower eukaryotic organisms (35). More than 26,400 proteins with HAMP domains exist in the SMART data base. These domains have been shown to play an active role in intramolecular signal transduction in prokaryotic sensor kinases. They are composed of about 50 amino acid residues each with two amphipathic helices (36-38) that probably rotate when the sensor domain of the protein is activated, as recently elucidated from nuclear magnetic resonance (NMR) analysis (39-40). Unlike bacterial HKs, which usually possess a single HAMP domain, fungal group III HKs have several consecutive HAMP domains. Examples of group III HKs are CaNik1p in *C. albicans* and Os1p in *N. crassa*.

Despite the larger numbers of HKs in filamentous ascomycetes than in yeasts, all of the ascomycetes contain virtually the same downstream histidine phosphotransfer proteins and RR proteins, suggesting extensive cross talk or redundancy among HKs (34). However, fungal hybrid sensor kinases are evidently more diverse than this initial estimate. For example, the *Cryptococcus neoformans* Tco2 hybrid sensor kinase contains two histidine kinase domains and two REC domains in a single polypeptide that cannot be classified into any of the 11 classes proposed by Catlett *et al.* (2003) (26).

1.3.7 Group III histidine kinases as a target for antifungals

Several chemical classes of fungicides (Fig. 1.4), which are known for their activity on filamentous fungi, such as phenylpyrroles (fludioxonil), dicarboximides (iprodione), and the polyketide secondary metabolites of ambruticins, have been shown to target the osmotic stress signal transduction pathway (Fig. 1.3) (41-43). The compounds exert their antifungal effects by activating the Hog1 MAPK module, resulting in the accumulation of both glycerol and free fatty acids. In the absence of high external osmolarity, artificial induction of excess intracellular glycerol is assumed to cause the leakage of cellular contents and ultimately results in cell death (44).

Heterologous functional expression of CaNik1p and other group III HKs in *S. cerevisiae* has enabled the transfer of sensitivity to antifungals activating the Hog1 MAPK module establishing the function of these HKs as mediators for antifungal activity (45-48) and providing an easy tool for testing the effects of genetic manipulation of the HKs. *S. cerevisiae* is usually resistant to these fungicides because of its lack of group III HKs (34).

In Hik1p, a group III HK from *Magnaporthe grisea*, phosphate acceptance on both the conserved histidine and aspartic acid residues in the HisKA and the receiver domains is essential for susceptibility to phenylpyrroles and ambruticin VS4 (45-46, 48). Additionally, mutations in the HAMP domains of several group III HKs are frequently associated with fungicide resistance, showing the dependence of fungicide activity on these domains (47, 49). However, whether the group III HKs are direct or indirect targets for the antifungals inducing the Hog1 MAPK module has not been experimentally demonstrated.

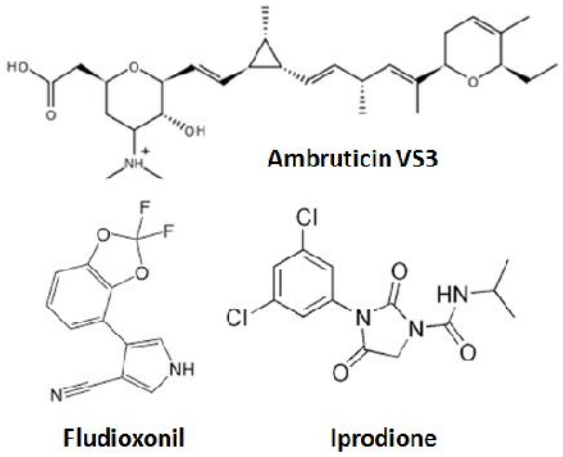


Fig. 1.4: Structures of various antifungals inducing the Hog1 MAPK module.

1.3.8 The histidine kinase CaNik1p from *C. albicans*

CaNik1p is considered to be a cytosolic enzyme, as it lacks the indicative hydrophobic amino acids for membrane-spanning domains (50). The protein is not essential for survival, and a gene deletion mutant can be generated (50-52). CaNik1p plays an important role in hyphal formation of *C. albicans* on solid media (25, 51). Additionally, the deletion mutant of *CaNIK1* has been found to be less virulent in a mouse model of systemic candidiasis (25).

CaNik1p belongs to group III HKs (34). Based on homology with other HKs, the histidine residue H510 and the aspartate residue D924 in CaNik1p (Fig. 1.5) have been identified as the essential phosphorylatable residues for the HisKA and the REC domains, respectively (52). The ATP-binding domain, HATPase_c, of CaNik1p comprises characteristic N and G1 boxes, with two asparagine residues (N623 and N627) and two glycine residues (G663 and G665), respectively (52). Both boxes are known to be essential for ATP binding (53). Mutation of a single Asn residue in the N box is associated with complete inhibition of ATP binding in the bacterial HK EnvZ (54). In addition, mutation of the two Gly residues of the G1 box leads to the same effect in the HK ETR1 in *Arabidopsis thaliana* (55).

CaNik1p has been described as possessing four full-length amino acid repeats of 92 amino acids each and one repeat truncated to 72 amino acids (52). Originally, one HAMP domain was allocated to each of these repeats. However, recently, the sequences of about 6500 HAMP domains from ca. 5500 distinct proteins have been analyzed leading to a new classification scheme with 4 clusters of canonical sequences and 3 more divergent clusters (38). We have applied this scheme to the HK CaNik1p, i.e., to the amino acid repeats from CaNik1p, and have identified nine divergent HAMP domains (47). To date, the role of the HAMP domains in controlling signal transduction downstream of group III HKs is unclear. Mutational analysis of the various domains of CaNik1p, being a group III HK, would be of interest in order to determine the way that the domains affect the fungicidal mechanism of compounds activating the Hog1 MAPK module.

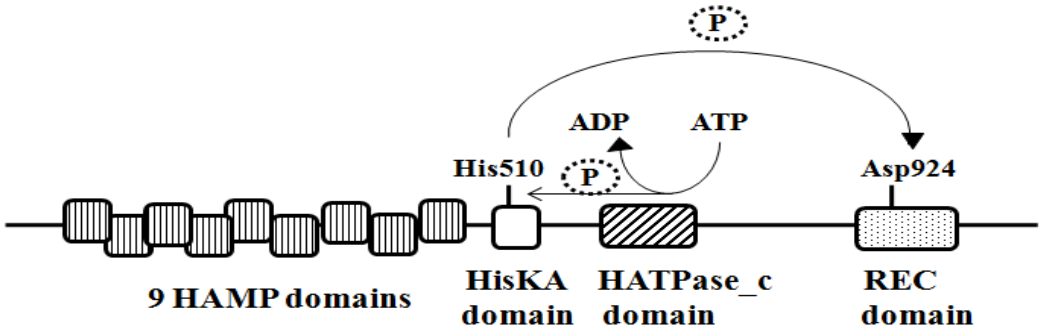


Fig. 1.5: Various domains of CaNik1p and their role in the phosphorylation of the protein.

1.4 Strategies for genetic manipulation of *C. albicans*

The *C. albicans* genome consists of eight chromosomes (historically named 1-7 and R) that constitute a haploid genome size of 14,851 kilobases (kb), containing 6,419 open reading frames (ORFs) longer than 100 codons (56).

In 2004, *C. albicans* was selected by Jones and coworkers as the first eukaryotic pathogen for genome sequencing (57). They used the diploid genome of the widely-used clinical isolate, SC5314, to sequence the genome of *C. albicans* (58). Thereafter, sequences of the majority of the *C. albicans* genes were available, making gene targeting in this organism much easier, faster, more efficient, and precise. Soon after, the Candida Genome Database (CGD) was established (59) based on the genome sequence of *C. albicans* enriched with published literature on *C. albicans* (57). The CGD (www.candidagenome.org) is a freely available valuable resource for researchers in the field, as it provides a reliable source of organized data, tools for data analysis, and information about the current research community.

Molecular analysis of *C. albicans* is particularly challenging for many reasons. First, *C. albicans* has no clearly defined sexual cycle (8). Second, the organism is diploid (58, 60), and in most cases, both alleles of the gene must be manipulated. Third, contrary to the most studied yeast (*S. cerevisiae*), *C. albicans* lacks natural plasmids, such as the 2-µm plasmid, for use in transformation and shows slower transformation and recombination frequencies, making the manipulation of both alleles of a gene more challenging (61-62). Fourth, the codon CUG is translated abnormally by *C. albicans* as serine rather than leucine (63), and it is found at least once in approximately two-thirds of the ORFs (56). Therefore, when using marker gene sequences from other organisms for gene manipulation in *C. albicans*, or in cases of the heterologous expression of *C. albicans* genes, the codons need to be optimized. Fifth, the use of selectable markers has been particularly challenging, because some markers, e.g., the *URA3* gene, have effects on the virulence of the pathogen (64). In addition, *C. albicans* shows natural resistance to drugs such as G418, hygromycin B, and cycloheximide, which are used for selection in *S. cerevisiae* (61).

Previously, mutations were randomly introduced into the *C. albicans* genome by exposure to UV or chemical mutagens. These methods led to undesirable mutations that could not be repaired (57).

Fonzi and Irwin used a clinical isolate of *C. albicans* (SC5314) to make a strain (CAI4) that was auxotrophic for growth on uridine-deficient media (65). They disrupted both copies of the *URA3* gene in *C. albicans* by using the imm434 region of the λgt10 bacteriophage. This allows the use of *URA3* as a selectable marker when genes are disrupted. The method is popularly known as the ‘‘URA blaster method’’. In this technique, the gene of interest is disrupted by using a cassette that carries the *URA3* gene flanked by two *hisG* sequences from *Salmonella typhimurium*. After the initial transformation, the transformants are selected on uridine-deficient media. Because two direct repeats of the *hisG* sequence in close proximity are unstable in the genome, spontaneous intrachromosomal recombination events can occur between these *hisG* sequences. Such recombination events will excise the *URA3* gene and leave behind one copy of the *hisG* sequence in the genome. The loop out event allows the recycling of the *URA3* marker for the disruption of the second allele. The use of *URA3* is specifically advantageous, because it can also be counter-selected. Therefore, in the next step, the cells that excise the *URA3* gene by a recombination event between the *hisG* direct repeats can be selected on 5-FOA (5-fluoroorotic acid), whereby 5-FOA is converted to the toxic form (5-flurouracil) in strains expressing the functional *URA3* gene. An additional second

transformation step potentially generates cells that are null for the gene of interest (57) (Fig. 1.6A).

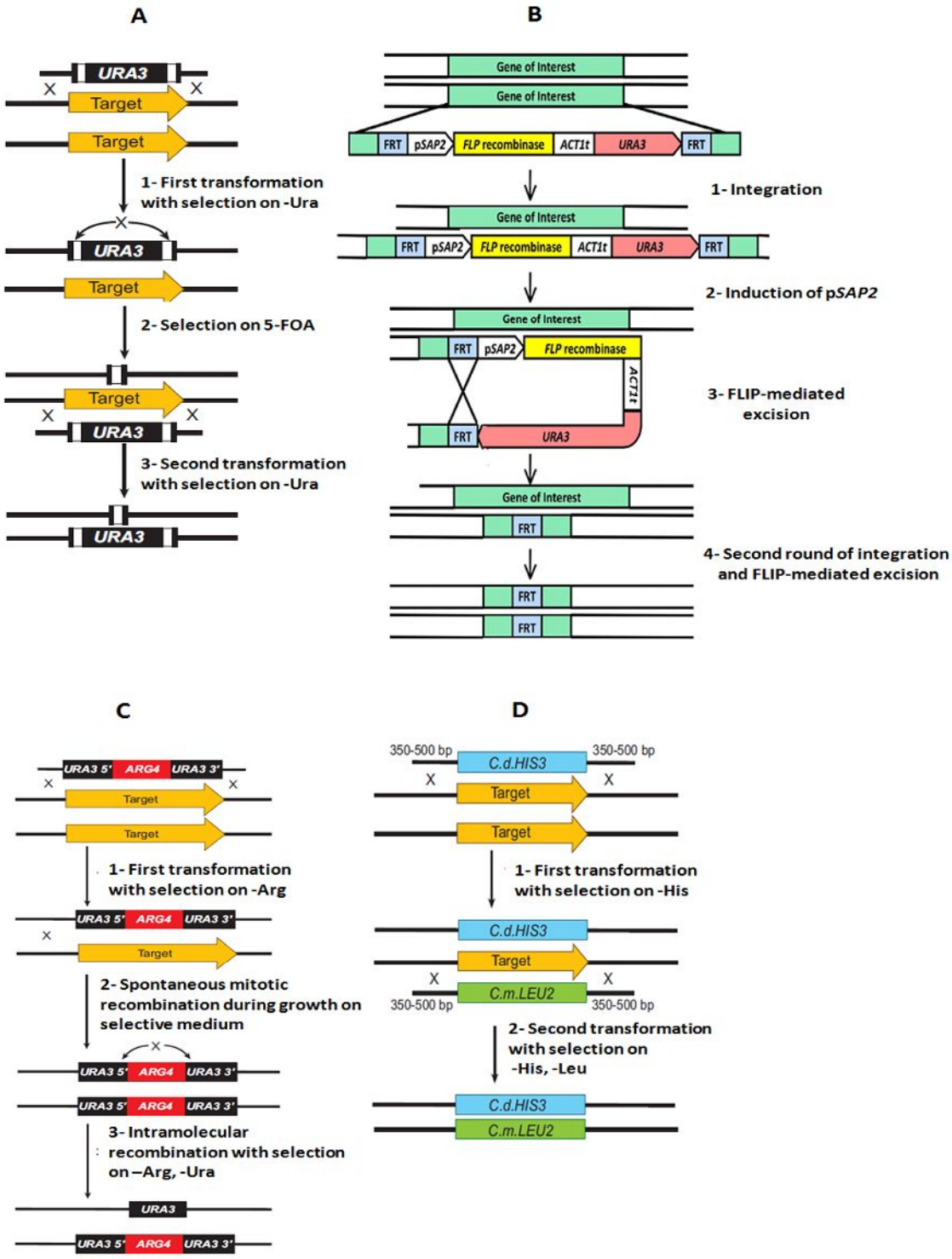


Fig. 1.6: Schematic diagram of some gene deletion cassettes that are used in *C. albicans* (8, 57). A) The URA blaster method, B) the URA flipper method, C) the UAU1 method, and D) the method developed by Noble and Johnson (66).

The URA blaster method has many drawbacks (57). First, the CAI4 strain has been shown to have defective expression of 14 different proteins when compared with its parent, SC5314 (67). Second, exposure to 5-FOA in the second step is potentially mutagenic and can introduce chromosomal rearrangements (68). Third, this method leaves a single copy of *URA3* at the locus of the gene of interest. Researchers have discovered that the expression of *URA3* at ectopic loci in the genome can affect the virulence of *C. albicans* (64, 67), and overall, the change in the chromosomal location of the *URA3* gene affects *C. albicans* Ura3 activity, hyphal morphogenesis, adherence, and lethality in mice (69). Phenotypic changes in about 30 % of the contemporary published papers have been, upon re-evaluation, attributable to the expression of *URA3* at an ectopic location in the genome and are not caused by the deletion of the gene of interest *per se* (67). Fourth, when using the URA blaster method for gene manipulation, the recombination between *hisG* repeats leaves a foreign sequence (one copy of the *hisG* sequence) in the genome. In instances when a promoter region of an adjacent gene is within or just following the termination region of the gene of interest, a foreign sequence that is left behind could be detrimental as it might affect the expression of the adjacent gene (57).

As a result of the problems associated with the URA blaster method, alternative versions of the URA blaster cassette or entirely new deletion cassettes were developed. Morschhauser *et al.*, 1999 introduced the use of the *FLP* recombinase system to make a recyclable *URA3* cassette (‘‘URA flipper’’) (70). In this method (Fig. 1.6B), the *URA3* marker is flanked by two direct repeats of the *Flp* recombinase recognition site (*FRT*). The expression of the *FLP* recombinase gene is controlled by the inducible *SAP2* promoter (secreted aspartyl proteinase family 2). After transformation, the *SAP2* promoter is activated, and *FLP* is expressed. To activate the *SAP2* promoter, the cells are grown in medium containing yeast carbon base and bovine serum albumin at pH 4.0. The expressed *Flp* recombinase then recognizes the *FRT* sites and excises the *URA3* gene by homologous recombination, making the auxotrophic marker available for the second transformation. This method avoids the use of 5-FOA and its associated mutagenic potential to counter-select for the *URA3* gene. However, similar to the URA blaster cassette, the URA flipper cassette also leaves behind a foreign sequence (a single *FRT* sequence) in the genome, at the locus of the gene of interest. Nevertheless, the foreign sequence left behind is extremely small, only 34 bp (57), in comparison with that of the *hisG* sequence (1149 bp) (65).

Enloe and coworkers constructed a UAU1 cassette (Fig. 1.6C) for gene deletion in the parent strain BWP17 (auxotrophic for *URA3*, *ARG4*, and *HIS1*) (71). The UAU1 cassette allows the disruption of both copies of the gene of interest within a single transformation step.

The UAU1 cassette comprises the *URA3* gene disrupted by the *ARG4* gene, but the 5` end sequence of the *URA3* gene carries a 530 bp sequence homologous to its 3`end sequence. Therefore, when transformed into the genome, recombination loops out the *ARG4* gene and makes a functional copy of *URA3*. This unique feature of the UAU1 cassette allows the selection of rare instances in which both alleles of the gene are replaced by mitotic recombination or gene conversion in a single transformation step. These rare double-deleted cells are detected by the presence of both *ARG4* and *URA3* markers (57). However, the potential homozygous deletion mutants need to be confirmed to rule out possible allelic triplications, in which a wild-type allele is retained in spite of two alleles having been replaced by the cassette (57). This condition might arise via known triplicated alleles in the genome, an increase in ploidy, tandem duplications, or translocations (71).

Noble and Johnson constructed parent strains that were auxotrophic for *LEU2*, *HIS1*, and *ARG4* nutritional markers. The main strains constructed were: SN87 (auxotrophic for *LEU2* and *HIS1*), SN95 (auxotrophic for *HIS1* and *ARG4*), and SN152 (auxotrophic for *LEU2*, *HIS1*, and *ARG4*) (66). All these strains were constructed via the URA blaster method except that they have one copy of *URA3* expressed at the native locus. Employment of these strains allows *C. albicans* genes to be disrupted without the use of the *URA3* marker. These markers are not recyclable and are left at the locus of the gene of interest (Fig. 1.6D). Extensive tests indicate that the expression of any of the above-mentioned nutritional markers in ectopic genomic locations does not affect the virulence of *C. albicans* (57). The use of heterologous marker genes from *C. maltosa* or *C. dubliniensis* strains has also been initiated when disrupting genes in *C. albicans* to decrease recombination events at the endogenous locus of the marker gene.

Some researchers have chosen to avoid entirely the use of nutritional markers to manipulate the *C. albicans* genome. This is necessary if the genes of interest are in pathways affected by nutritional status (57).

Köhler and coworkers have developed a strategy involving inosine monophosphate dehydrogenase (*IMH3*) as a selectable marker for *C. albicans* genetic manipulation (72). IMH3 directs the *de novo* synthesis of GMP and is inhibited by mycophenolic acid (MPA). Köhler *et al*. overexpressed *IMH3* from a plasmid in the strain CAI4 and found that the successful transformants were far more resistant to MPA compared with the wild-type strains. However, the method was not developed to the level of chromosomal integration (57). Wirsching and coworkers used MPA resistance as a dominant selectable marker for chromosomal gene disruptions; they employed a form of the *IMH3* gene that was mutated so

as to avoid reintegration of the fragment into the normal chromosomal copy of *IMH3* in *C. albicans* (73). They adapted the URA flipper strategy to this system, whereby *FRT* recombination sites were positioned to flank the *FLP* recombinase gene (driven by the *SAP2* promoter) and the *IMH3* gene (replacing URA3 selectable marker) to generate a ‘‘MPA^R flipper’’ cassette (57).

The group of Morschhauser used the drug nourseothricin (NST) as a dominant selection marker for gene disruption (74). They introduced, into *C. albicans* cells, the streptothricin acetyl transferase (*SAT1*) gene (from bacterial transposon Tn1825), which confers resistance to the drug NST. This method, called the *SAT1* flipper, circumvents problems related to the use of nutritional markers and auxotrophic host strains. In addition, the *FLP* recombinase system has been included in the *SAT1* flipper cassette to allow for the recycling of the selection marker *SAT1* without the use of mutagenic substances that might lead to undesirable mutations in the genome. The homozygous mutants obtained after two rounds of insertion and recycling of the *SAT1* flipper cassette differ from the wild-type parental strain only in the absence of the target gene (replaced by a single *FRT* sequence) and can be used for the inactivation of additional genes and the generation of complemented strains by employing the same strategy. Because of the previously mentioned advantages of the *SAT1* flipper cassette, it was adopted in this work to delete *CaNIK1* in *C. albicans*; its principle is described in detail in 4.11.3 (Page 74).

2 Aims of the work and experimental design

An urgent need exists to develop new antifungals with new targets that are absent in humans, as most of the antifungals that are present on the market are associated with problems of either toxicity and/or resistance. Group III HKs, e.g., CaNik1p from *Candida albicans*, are part of TCST systems that are absent in mammalian cells. Moreover, they are present in most pathogenic fungi and have been reported to play an essential role in the virulence of these fungal species. Noteworthy, they have been found to be fundamental for conferring sensitivity to many antifungals that are known to exert their effect by inducing the Hog1 MAPK module. Therefore, group III HKs represents an attractive approach for developing a new promising class of antifungal agents. Despite numerous reports that have confirmed the basic role of these kinases for fungicidal activity, many questions remain unanswered. For example, do the antifungals interact directly with the class III HKs? If so, do they interfere with their kinase activity? On the other hand, in *C. albicans*, CaNik1p plays an important role in hyphal formation (virulence factor) on solid media. However, the role of the various domains of the protein for hyphal formation has not been investigated as yet.

Therefore, the aims of the present work are:

1- Investigation of the relevance of various domains of CaNik1p for fungicidal activity and for activation of the Hog1 MAPK module after fungicidal treatment.

2- Study of the relevance of various protein domains for the kinase activity of the protein and investigation of the correlation between such kinase activity and the antifungal mechanism.

3- Investigation of whether group III HKs, with CaNik1p as an example, are direct targets or mediators for the action of the antifungals, by using fludioxonil as a representative example.

4- Relevance of the various domains of CaNik1p for the phenotypic behavior of *C. albicans*, e.g., hyphal formation on solid medium.

To fulfil the above aims, the following methods were applied by using *Saccharomyces cerevisiae* as a model organism for the expression of *CaNIK1*:

1- Site-directed mutagenesis in various domains of CaNik1p including the conserved phosphorylatable residues of the HisKA and REC domains of the protein, in addition to the complete deletion of the characteristic domains of group III HKs, the HAMP domains.

2- Transformation of *S. cerevisiae* with various plasmids harboring the mutated variants of *CaNIK1*.

3- Testing the resultant transformants for:

- Antifungal susceptibility in comparison with the sensitive transformant expressing the wild-type *CaNIK1*.
- Phosphorylation of the MAPK Hog1p, as an indicator for the activation of the Hog1 MAPK module, by Western blot after fungicidal treatment of various transformants.

4- Expression and purification of the His-tagged CaNik1p and its mutated variants followed by investigation of their *in vitro* kinase activity either by the Kinase-Glo plus kit or by incubation with radioactive [γ-^{32}P] ATP.

5- Study of the direct interaction between the purified CaNik1p and fludioxonil by using Saturation Transfer Difference-NMR.

6- *In vitro* kinase assay of the purified CaNik1p in the presence of fludioxonil.

7- Transfer of the mutated variants of *CaNIK1* to *C. albicans* via deletion of both alleles of the *CaNIK1* in *C. albicans* and reintegration of these mutated variants. For such a genetic manipulation in *C. albicans*, the *SAT1* flipper cassette has been used.

3 Materials and Methods

3.1 Materials

3.1.1 Solutions & Buffers

3.1.1.1 General solutions

10X PBS	
NaCl	80 g/l
KCl	2 g/l
Na_2HPO_4	14.4 g/l
KH_2PO_4	2 g/l
pH	7.3

Washing buffer	
NaH_2PO_4 x H_2O	0.52 g/l
Na_2HPO_4 x $2H_2O$	8.80 g/l
NaCl	2.83 g/l
KCl	0.372 g/l
pH	7

Lysis buffer A (for routine cell disruption)

Prepared by dissolving one tablet of complete ULTRA protease inhibitor (Roche) and one tablet of PhosSTOP Phosphatase Inhibitor Cocktail (Roche) in 10 ml washing buffer.

Lysis buffer B (for cell disruption prior to purification of CaNik1p and its mutants)

Prepared by dissolving two tablets of complete ULTRA protease inhibitor, Mini, EDTA-free (Roche) and one tablet of PhosSTOP Phosphatase Inhibitor Cocktail in 8 ml washing buffer.

3.1.1.2 For Protein methods

SDS-PAGE gels	Separating gel (% Acrylamide)		Stacking gel (4 % Acrylamide)
	7.5	12.5	
Deionized H_2O	6 ml	4 ml	2.9 ml
Lower Tris (1.5 M Tris-HCl, pH 8.8)	3 ml	3 ml	-
Upper Tris (0.5 M Tris-HCl, pH 6.8)	-	-	1.25 ml
Acrylamide/Bis-acrylamide stock solution (30 %, mixed in ratio 37.5:1) (Rotiphorese Gel 30, Roth)	3 ml	5 ml	0.85 ml
20 % (w/v) SDS	60 µl	60 µl	25 µl
10 % (w/v) APS	60 µl	60 µl	25 µl
TEMED	15 µl	15 µl	7 µl
Above amounts are sufficient for preparation of 2 minigels.			

5x Laemmli buffer	
Glycerin	2 ml
20 % (w/v) SDS	5 ml
Tris-HCl, pH 6.8, 0.5 M	2.5 ml
β-Mercaptoethanol	100 µl
Bromophenol blue	50 mg

Coomassie Staining solution	
Coomassie brilliant R250	0.7 g
Ethanol	25 ml
Acetic acid	8 ml
Water	67 ml

Destaining solution	
Ethanol	40 %
Acetic acid	10 %

BCA-Reagent A	
BCA	10 g/l
Na_2CO_3	17.1 g/l
Na_2Tartrat. $2H_2O$	1.9 g/l
$NaHCO_3$	9.5 g/l
pH	Adjusted to 11.25 with 10 N NaOH

BCA-Reagent B	
$CuSO_4.5H_2O$	40 g/l

10x TGS buffer	
Tris-HCl	25 mmol/l
Glycine	192 mmol/l
SDS	0.1 % (v/v)
pH	8.3

TBS-T Buffer	
Tris-HCl	20 mmol/l
NaCl	140 mmol/l
Tween 20	0.1 % (v/v)
pH	7.4

Blot buffer:	
Tris base	3,03 g/l
Glycine	14,4 g/l

2x NPI buffer	
$NaH_2PO_4.2H_2O$	7.8 g
NaCl	17.54 g
pH	8
Water to	500 ml

Imidazole Stock solution (1 M)	
Imidazole	6.804 g
Water to	100 ml (pH adjusted to 8)

Buffers for purification of His-tagged CaNik1p using Ni^{+2}-agarose beads			
	NPI-10 (equilibration buffer)	**NPI-50 (washing buffer)**	**NPI-500 (elution buffer)**
2x NPI buffer:	25 ml	250 ml	25
Imidazole Stock solution (1 M)	0.5 ml	25 ml	25
Water to	50 ml	500 ml	50
Final concentration of imidazole (mM)	10	50	500

3.1.1.3 Kinase assay

10x Kinase buffer A	
Tris-HCl	400 mM
$MgCl_2.6H_2O$	200 mM
BSA	50 mg
pH	7.5
Water to	50 ml

ATP stock solution (10 mM)

100 mg of ATP powder (Sigma) was dissolved in 19.7 ml 1x kinase buffer A and the solution was divided into aliquots of 500 µl before storage at -20 ˚C.

8x Kinase buffer B	
Tris-HCl	50 mM
KCl	50 mM
$MgCl_2$	5 mM
Dithiothreitol	2 mM
pH	7.5

Flow-through buffer (used as a negative control in the *in vitro* kinase assay via the Kinase-Glo Plus kit)

500 µl of the 10^{-4} diluted imidazole stock solution (1 M) was added to 9.5 ml washing buffer to reach a final concentration of 0.005 mM imidazole.

Hot ATP stock solution

[γ-^{32}P] ATP (FP301, 370 MBq (100 µCi/10 µl), 3 µM ATP, Hartman Analytic) was diluted with 1x kinase buffer B (10 µl hot ATP + 90 µl 1x kinase buffer B) resulting in a final concentration of 1 µCi/1 µl (when diluted on the day of arrival) with a molar concentration of 0.3 µM for ATP in a total volume of 100 µl. The hot ATP stock solution was stored at -20 °C.

3.1.1.4 For molecular biology methods

EDTA (0.5 M, pH 8)

186.1 g disodium EDTA.2H_2O was added to 800 ml water and then stirred while the pH was adjusted to pH 8 by using NaOH pellets (about 20 g).

10x TE (Tris-EDTA) buffer	
Tris-HCl	100 mmol/l
EDTA	10 mmol/l
pH	8 or 7.5

50x TAE (Tris-acetate EDTA) buffer	
Tris base	242 g/l
EDTA (0.5 M) (pH 8)	100 ml
Concentrated acetic acid	57 ml

Ethidium bromide stock solution

12.5 mg ethidium bromide was dissolved in 10 ml water, and the solution was stored at 4 °C in a dark container.

Ethidium bromide working solution

For visualization of DNA under UV, 8.5 µl ethidium bromide stock solution was added to 20 ml agarose solution in TAE buffer (at 50 °C).

Breaking buffer	
Triton X-100	200 µl
20 % SDS	500 µl
NaCl	58.44 mg
Tris-HCl , pH 7.5, 1 M	100 µl
EDTA, 500 mM, pH 8	20 µl
Water	to 10 ml

2 M lithium acetate

10.2 g lithium acetate dihydrate was dissolved in 50 ml water (pH was adjusted to 8.4-8.9). The solution was sterilized by filtration through a Steriflip-NY Filter.

50 % PEG-3350

25 g PEG-3350 was dissolved in 12.5 ml water by heating in a microwave oven. The solution was made up to 50 ml with water before it was sterilized by filtration through the Steriflip-NY Filter.

Yeast transformation mixture (Must be freshly prepared)	
2 M lithium acetate	200 µl
50 % PEG-3350	800 µl
β-mercaptoethanol	7.7 µl
The solution was subjected to vortexing for proper mixing.	

Denatured salmon sperm DNA (10 mg/ml)

Salmon sperm DNA (10 mg) was dissolved in 1 ml filter-sterilized TE buffer (pH 8). The DNA solution was boiled for 5 min and chilled quickly on ice before being divided into aliquots of 50 µl and stored at -20 °C. Each aliquot could be used in the transformation of yeast for up to three freeze-thaw cycles.

3.1.1.5 Solutions of antimicrobial agents

Ampicillin Stock solution (Must be freshly prepared)

The ampicillin stock solution was prepared by dissolving 100 mg ampicillin powder in 10 ml sterile water.

Ampicillin working solution

1 ml ampicillin stock solution was added to 100 ml of LB broth or LB agar to give a

final concentration of 100 µg/ml.

Chloramphenicol Stock solution

The chloramphenicol stock solution was prepared by dissolving 340 mg chloramphenicol powder in 10 ml 100 % ethanol. The solution was stored at -20 °C in a dark container to avoid light degradation.

Chloramphenicol working solution

0.5 ml chloramphenicol stock solution was added to 100 ml of LB broth or LB agar to give a final concentration of 170 µg/ml.

Nourseothricin (NST) stock solution

The NST stock solution was prepared by dissolving 100 mg NST powder in 1 ml water. The solution was sterilized by filtration through a Minisart membrane filter before storage at –20 °C in aliquots of 250 µl.

NST working solutions

200, 20, 10, or 5 µl from the NST stock solution were added to 100 ml YPD broth or YPD agar to give a final concentrations of 200, 20, 10, or 5 µg/ml, respectively.

Stock solutions of antifungal agents

The antifungals fludioxonil (Fluka), iprodione (Fluka), and ambruticin Vs3 (produced as described and kindly provided by K. Gerth and R. Jansen (HZI, Braunschweig) (75)) were prepared as stock solutions in methanol at a concentration of 10 mg/ml and stored at -20 °C.

When fludioxonil was studied for its effect on the *in vitro* kinase activity of the CaNik1p, it was freshly prepared for each experiment as a stock solution in dimethylsulfoxide (DMSO) (10 mg/ml).

3.1.2 Media

All media were prepared in deionized water and sterilized by autoclaving unless otherwise specified. For the preparation of solid medium, 1.5 % agar was added to the liquid medium before autoclaving.

YPD broth

50 g YPD broth (Sigma) was dissolved in 1 liter water.

YPD agar

65 g YPD agar (Sigma) was dissolved in 1 liter water.

Drop out 1 solution (stored at RT)	
Adenine sulfate	400 mg
L-phenylalanine	500 mg
L-Glutamic acid	1 g
L-Aspartic acid	1 g
L-Threonine	2 g
Adenine Sulfate	400 mg
Water to	250 ml
For complete dissolution of the amino acids, the solution was stirred for 4 h, followed by sterilization via filtration through a Bottle-Top 500 ml Nalgene membrane filter.	

Drop out 2 solution (stored at 4 °C)	
L-Tryptophan	200 mg
L-Histidine HCl	200 mg
L-Arginine HCl	200 mg
L-Methionine	200 mg
L-Tyrosine	300 mg
L-Leucine	600 mg
L-Isoleucine	300 mg
L-lysine HCl	300 mg
L-Valine	1.5 g
L-Serine	4 g
Water to	1000 ml
For complete dissolution of the amino acids, the solution was stirred for 4 h, followed by sterilization via filtration through a Bottle-Top 500 ml Nalgene membrane filter.	

10x Galactose/Raffinose solution	
Galactose	200 g
Raffinose	100 g
Water to	1000 ml
For complete dissolution of the sugars, the solution was stirred for 4 h, followed by sterilization via filtration through a Bottle-Top 500 ml Nalgene membrane filter.	

SD-ura	
Yeast nitrogen base without amino acids	6.7 g
Dextrose	20 g
Deionized water	to 875 ml
The solution was sterilized by autoclaving and made up to 1 liter by the addition of 25 and 100 ml of sterile drop out 1 and 2 solutions, respectively.	

SG-ura	
Yeast nitrogen base without amino acids	6.7 g
Deionized water	to 775 ml
The solution was sterilized by autoclaving and made up to 1 liter by the addition of 25, 100, and 100 ml of sterile drop out 1, drop out 2, and 10x galactose/raffinose solutions, respectively.	

Uracil stock solution	
Uracil	100 mg
Water to	100 ml
The solution was sterilized by filtration through a Steriflip-NY Filter.	

Uracil working solution

800 µl from the uracil stock solution was added to 20 ml SD-ura for cultivation of the reference strains of *S. cerevisiae* BWG1-7a and BY4741 (auxotrophic strains for uracil).

LB medium	
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
pH	7
Water	to 1000 ml

NZY$^+$ broth	
NZ amine (casein hydrolysate)	10 g
Yeast extract	5 g
NaCl	5 g

pH	7
Water	to 945 ml
After autoclaving, the following supplements were added after sterilization via a Steriflip-NY filter:	
1 M $MgCl_2$	12.5 ml
1 M $MgSO_4$	12.5 ml
20 % glucose solution in water	20 ml

3.1.3 Kits

Kit	Company
Miniprep Qiagen plasmid DNA Extraction	Qiagen, Hilden, Germany
QIAquick Gel Extraction	Qiagen, Hilden, Germany
Kinase-Glo® Plus (V3771)	Promega
QuikChange site-directed mutagenesis	Strategene
Protino® Ni-NTA Agarose	Macherey Nagel, Düren, Germany
In-Fusion® HD Cloning	Clontech Laboratories
ECL, advance Western blotting detection	GE Healthcare

Tab. 3.1: Kits used in this study.

3.1.4 Software and Web interfaces

Software or Web interface	Used in
Adobe Photoshop 7.0 ME	Adjusting acceptable resolution of images
Aida image analyzer V:4.15	Opening the format files of the images obtained from the phosphorimage analyzer
BAS reader	Reading out image data from the phosphorimage analyzer
EasyWin	Documentation of agarose gels
End Note X3 and X6	Managing references in Microsoft Office Word
Excel 2007, Origin 6	Numerical data analysis and creating graphs
Geospiza's FinchTV	Chromatogram viewer of DNA sequences
Plasm 2.0.4.29	Drawing of plasmid maps

Software or Web interface	Used in
Gen5, Version 1.06, BioTek Instruments	Measurement of absorption and fluorescence
Image Reader, Fujifilm	Chemiluminescence detection
Chromeleon Software version 6.8, Dionex, Thermo Scientific	Data analysis of chromatograms in the LC-MS/MS
Xcalibur software version 2.1, Thermo Scientific	Analysis of data obtained from the mass spectrometer
Mascot Daemon-aided version 2.8, Matrix Science	Application that automates the submission of data files to Mascot server
Mascot server version 2.3.02, Matrix Science	Protein identification with MS data
Proteome Discoverer program Version 1.3, Thermo Scientific	Analysis of qualitative and quantitative proteomics data obtained from MS.
http://web.expasy.org/protparam/	Determination of MW and extinction coefficient of a protein
http://blast.ncbi.nlm.nih.gov/Blast.cgi	Blasting of DNA and protein sequences
http://tools.neb.com/NEBcutter2/	Determination of restriction sites in DNA sequences
http://web.expasy.org/translate/	Translation of DNA sequences
http://www.candidagenome.org/	Determination of sequences of various genes and the phenotypes associated with their expression in *C. albicans* Sc5314.
http://www.bioinformatics.org/sms/rev_comp.html	Conversion of a DNA sequence into its reverse, complement, or reverse-complement counterpart
http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/	Analysis of the properties of the designed primers.
http://www.ebi.ac.uk/Tools/msa/clustalw2/	Multiple alignment of DNA and protein sequences
http://domaindraw.imb.uq.edu.au./	Designing of schematic diagrams illustrating the presence and the position of one or more motifs or domains in certain protein

Software or Web interface	Used in
http://www.brenda-enzymes.org/	Information about properties of reference enzymes.
http://smart.embl-heidelberg.de/	To obtain information about various domains in the protein data base.
http://pfam.sanger.ac.uk/search	Identification of possible conserved domains in a protein.
http://www.ebi.ac.uk/Tools/msa/clustalw2/	Alignment of multiple sequences of proteins.

Tab. 3.2: Software and web interfaces used in this study.

3.1.5 Plasmids

Plasmid	Relevance and properties	Reference (source)
pYES2	Shuttle vector for transformation of *E. coli* and *S. cerevisiae.*	Invitrogen
pYES2-*CaNIK1*-TAG	Used in the construction for all mutants of the *CaNIK1*.	(47)
pYES2-*CaNIK1*(H510Q)	pYES2-*CaNIK1*-TAG containing point mutation in the codon of His510 (CAT to CAA; His to Gln).	This study
pYES2-*CaNIK1*(D924N)	pYES2-*CaNIK1*-TAG containing point mutation in the codon of Asp924 (GAT to AAT; Asp to Asn).	This study
pYES2-*CaNIK1*(H510Q, D924N)	pYES2-*CaNIK1*-TAG containing point mutations in the codons of His510 (CAT to CAA; His to Gln) and Asp924 (GAT to AAT; Asp to Asn).	This study
pYES2-*CaNIK1*(N627D)	pYES2-*CaNIK1*-TAG containing point mutation in the codon of Asn627 (AAT to GAT; Asn to Asp).	This study
pYES2-*CaNIK1*(G663A, G665A)	pYES2-*CaNIK1*-TAG containing point mutation in the codons of Gly663 (GGA) and Gly665 (GGT) into GCA (Ala) and GCT (Ala) respectively.	This study
pYES2-*CaNIK1*ΔHAMP	pYES2-*CaNIK1*-TAG containing deletion of the HAMP domains (sequence encoding the amino acids 63-485 in the CaNik1p).	This study
pYES2-*CaNIK1*ΔHAMPup	pYES2-*CaNIK1*-TAG containing deletion of HAMP domains and its upstream part in the *CaNIK1* (sequence encoding the amino acids 1-485 in the CaNik1p).	This study
pYES2-*CaNIK1*ΔHAMP(H510Q)	pYES2-*CaNIK1*ΔHAMP containing point mutation in the codon of His510 (CAT to CAA; His to Gln).	This study
pYES2-*CaNIK1*ΔHAMPup (H510Q)	pYES2-*CaNIK1*ΔHAMPup containing point mutation in the codon of His510 (CAT to CAA; His to Gln).	This study
pYES2-*CaNIK1Δ*224-315*Δ*327-418 aa	pYES2-*CaNIK1*-TAG containing deletion of sequences encoding the amino acids 224-315 and 327-418 in the CaNik1p.	(47)
pSFS2A	Contains the *SAT1* flipper cassette.	(74)
pNIK	Contains the *CaNIK1* deletion cassette.	This study

Tab. 3.3: Plasmids used in this work.

3.1.6 Oligonucleotides

Name	Sequence (5' → 3')	Relevance
F2Gln	CTAGCGAACATGTCGCA**A**GAGATACGTACACC	Construction of point mutation in the HisKA domain at His510
R2Gln	GGTGTACGTATCTC**T**TGCGACATGTTCGCTAG	
AAsnF	GATGTGGTGTTGATG**A**ATGTGCAAATGCCTGTAATG	Construction of point mutation in the REC domain at Asp924
AAsnR	CATTACAGGCATTTGCACAT**T**CATCAACACCACATC	
TAsnF	CTTAACTTGGCTGGT**G**ATGCTATTAAGTTTAC	Construction of point mutation in the HATPase_c domain at N box (N627)
TAsnR	GTAAACTTAATAGCAT**C**ACCAGCCAAGTTAAG	
F1ATP	GTGTTAGCGACACGG**C**AATAG**C**TATAGAGAAAGACAA	Construction of double point mutations in the HATPase_c domain at the G1box (G663 and G665)
R1ATP	TTGTCTTTCTCTATA**G**CTATT**G**CCGTGTCGCTAACAC	
HMPF1	AGGGAATATT**AAGCTT**ATGAACCCCACTAAAAAACCACG	Deletion of the HAMP domains (sequence encoding the amino acids 63-485 in the CaNik1p)
HMPR1	GTTCGCGTTTTTGGATTTTTCTAG	
HMPF2	TCCAAAAACGCGAACAGGAATACTGCGGCTAGAGAAGCTG	
HMPR2	GCTCGGTACC**AAGCTT**TCAGTGGTGATGGTGATGATGTCC	
HisKF2	GAATGC**AAGCTT**ATGAGGAATACTGCGGCTAGAG	Deletion of the HAMP domains and its upstream part in the *CaNIK1* (sequence encoding the amino acids 1-485 in the CaNik1p)
HisKR	GTATTG**TCTAGA**GTCAGTGGTGATGGTGATGATG	

Tab. 3.4A: Primers used for the construction of mutated variants of *CaNIK1*.

Name	Sequence (5' → 3')	Target sequence
F1	CCTCTATACTTTAACGTCAAGG	-96 to -75
R2Gln	GGTGTACGTATCTCTTGCGACATGTTCGCTAG	1535-1514
Fseq	GGGAATCGCTTCAAAGGAATAC	1442-1463
Rseq	CGAATCTTCTCCGTAGCTTCG	2819-2800
F4	CTCGTCAATCAGAAACTTGCAGTTAG	2647-2672

Tab. 3.4B: Primers used for sequencing of *CaNIK1* in the pYES2 vector.

Name	Sequence (5' → 3')	Relevance
F1NIK	CTATAGGGCGAATTG**GGTACC**ATAGAGTGGGACATAGGTGGAG	Amplification of the *CaNIK1*-upstream sequence.
R1bNIK	AACTTCCTCGAGGGG**GGGCCC**TGGAGAGAGAGGGAGAAACTCG	
F2NIK	CTAGAGCGGCCGCCA**CCGCGG**CTAACGACTCAAGCGTCAGCTTG	Amplification of the *CaNIK1*-downstream sequence
R2NIK	AAAGCTGGAGCTCCA**CCGCGG**GGTCACGTGATGTTGCATAAACTG	
F1SAT	TTGTAGCCACTGAAACCACG	Check for the existence of the *SAT1* flipper cassette
R1SAT	TCTCCCCCTTCACACTTCAC	
F4NIK	**GGGCCC**CCCCTCGAGGAAGTT	Amplification of pNIK by inverse PCR for the design of the *CaNIK1* integration cassette
R3NIK	TTTAGTGGGGTTCATTGGAGAGAGAGGGAGAAACTCG	
F3NIK	ATGAACCCCACTAAAAAACCACG	Amplification of *CaNIK1*-TAG and its mutated variants for the design of the *CaNIK1* integration cassette
R5NIK	AACTTCCTCGAGGGG**GGGCCC**TCAGTGGTGATGGTGATGATG	

Tab. 3.4C: Primers used for the construction of *CaNIK1* deletion and integration cassettes.

Tab. 3.4: Oligonucleotides used in this study. Bold bases represent the restriction sites. Single bold underlined bases are those that were used to introduce the point mutations. Dot-underlined bases are the overlapping bases that allowed the ligation of DNA fragments via the In-Fusion enzyme. Double underlined bases represent the initiation codon ATG. The letters F and R in the names of the primers refer to the primers being forward and reverse, respectively.

3.1.7 Strains

Species and strain designation	Genotype	Transformed with	Reference (Source)
TOP10	*F- mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galUgalKrpsL (StrR) endA1 nupGλ-*	pYES2-*CaNIK1*-TAG	(47)
XL1-Blue supercompetent cells	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proABlacI*qZΔ*M15* Tn*10* (Tetr)]	Point mutated variants of the *CaNIK1* harbored in the pYES2	Stratagene
Stellar™ Competent Cells	*F–, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA - argF) U169, Δ (mrr- hsdRMS - mcrBC), ΔmcrA, λ–*	- pYES2-*CaNIK1*ΔHAMP - pYES2-*CaNIK1*ΔHAMPup -pNIK	Clontech
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A*sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	pSFS2A	(74)

Tab. 3.5: *E. coli* strains used in this study.

Species and strain designation	Genotype	Transformed with	Reference (Source)
S. cerevisiae			
BWG1-7a	*Mat* a *ura3-52 leu2-3,112 his4-519 ade1-100*	-	(76)
YES	BWG1-7a	pYES2	This study
NIK	BWG1-7a	pYES2-*CaNIK1*-TAG	(47)
H510	BWG1-7a	pYES2-*CaNIK1*(H510Q)	This study
D924	BWG1-7a	pYES2-*CaNIK1*(D924N)	This study
N627	BWG1-7a	pYES2-*CaNIK1*(N627D)	This study
G1	BWG1-7a	pYES2-*CaNIK1*Δ(G663A, G665A)	This study
ΔHa	BWG1-7a	pYES2-*CaNIK1*ΔHAMP	This study
ΔHupa	BWG1-7a	pYES2-*CaNIK1*ΔHAMPup	This study
ΔHaH510	BWG1-7a	pYES2-*CaNIK1*ΔHAMP(H510Q)	This study
ΔHupaH510	BWG1-7a	pYES2-*CaNIK1*ΔHAMPup(H510Q)	This study

Species and strain designation	Genotype	Transformed with	Reference (Source)
S. cerevisiae			
ΔH3H4	BWG1-7a	pYES2-*CaNIK1Δ*224-315*Δ*327-418 aa	(47)
BY4741	*Mat* a *his3*Δ 1; *leu2*Δ 0; *met15*Δ 0; *ura3*Δ 0	-	(77)
ΔHb	BY4741	pYES2-*CaNIK1*ΔHAMP	This study
ΔHupb	BY4741	pYES2-*CaNIK1*ΔHAMPup	This study
ΔHbH510	BY4741	pYES2-*CaNIK1*ΔHAMP(H510Q)	This study
ΔHupbH510	BY4741	pYES2-*CaNIK1*ΔHAMPup(H510Q)	This study
Δssk1	BY4741, *YLR006c::kanMX4*	-	(78)
Δpbs2	BY4741, *YJL128c::kanMX4*	-	(78)
Δhog	BY4741, *YLR113w::kanMX4*	-	(78)
NIKb Δhog	Δhog	pYES2-*CaNIK1*-TAG	This study
H510bΔhog	Δhog	pYES2-*CaNIK1*(H510Q)	This study
N627bΔhog	Δhog	pYES2-*CaNIK1*(N627D)	This study
G1bΔhog	Δhog	pYES2-*CaNIK1*Δ(G663A, G665A)	This study
HDbΔhog	Δhog	pYES2-*CaNIK1*(H510Q, D924N)	This study
ΔHbΔssk1	Δssk1	pYES2-*CaNIK1*ΔHAMP	This study
ΔHbΔpbs2	Δpbs2	pYES2-*CaNIK1*ΔHAMP	This study
ΔHbΔhog	Δhog	pYES2-*CaNIK1*ΔHAMP	This study
ΔHupbΔssk1	Δssk1	pYES2-*CaNIK1*ΔHAMPup	This study
ΔHupbΔpbs2	Δpbs2	pYES2-*CaNIK1*ΔHAMPup	This study
ΔHupbΔhog	Δhog	pYES2-*CaNIK1*ΔHAMPup	This study
C. albicans			
Sc5314	Sequenced wild type	-	(79)
NIKa *	*nik1-1*Δ::*SAT1-FLIP* /*nik1-2*Δ:: *SAT1-FLIP*	*CaNIK1* deletion cassette, integrated in both alleles of the *CaNIK1* gene	This study
NIKhom2 *	*nik1-1*Δ:: *FRT* /*nik1-2*Δ::*FRT*	-	This study

Tab. 3.6: *S. cerevisiae* and *C. albicans* strains used in this study. * The genotype of the strain has been confirmed only by PCR.

3.2 Methods

3.2.1 Maintenance of cultures

Overnight cultures were prepared from separate colonies and processed as follows:

3.2.1.1 Long term storage:

Overnight cultures (20 ml) were centrifuged at 5000 rpm for 5 min. The supernatant was rejected, and the cell pellet was resuspended in the remaining supernatant. In a sterile cryotube, 625 µl from the cell suspension was mixed with 375 µl 80 % sterile glycerol solution to give a final concentration of 30 % glycerol. The glycerol stock tubes were then stored at -80 ˚C.

3.2.1.2 Cryo-stocks for routine cultivation of cultures

The overnight culture was distributed in cryotubes, so that each contained 2 ml, and stored at -20 ˚C.

3.2.2 Measurement of growth of cultures

3.2.2.1 OD measurement

Optical density at 620 nm (OD_{620nm}) was determined in sample volumes of 180 µl in 96-well microtiter plates by using the scanning microplate spectrophotometer µQuant.

3.2.2.2 Cell number counting

Cells were counted by using a Neubauer improved hemocytometer cell counting chamber (Fig. 3.1). The chamber consisted of nine large squares, each occupying 0.1 µl and having an area of 1 mm^2. The square in the center was divided into 5 × 5 small squares (each 0.04 mm^2). The chamber was filled with 10 µl cell suspension, and the cells were counted under the microscope in five small squares on the same diagonal within the central large square. The resulting number was multiplied by 5×10^4 to obtain the cell number per ml. To avoid too high a cell density, the cell suspensions were diluted in phosphate-buffered saline (PBS), and the dilution factor was included in the calculation of the cell density.

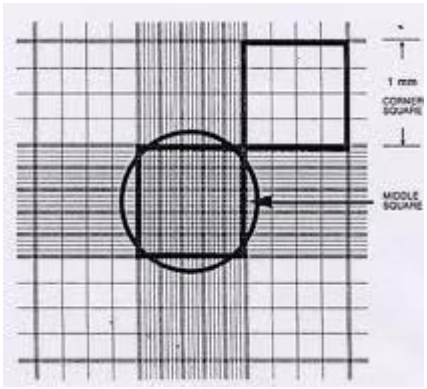


Fig. 3.1: Schematic diagram of the hemocytometer counting chamber (80).

3.2.3 Cultivation in Erlenmeyer flasks

In all experiments, the cultivation of organisms was performed in baffled Erlenmeyer flasks that contained medium to not more than 1/5 their volume. The culture flasks were shaked at 160 rpm in the shaker incubator Multitron Standard, Infors HT.

3.2.4 Cultivation of bacterial strains

E. coli strains were cultivated in LB or NZY^+ medium at 37 °C. To select and maintain the transformed cells, ampicillin or chloramphenicol was added to the medium at final concentrations of 100 µg/ml or 170 µg/ml, respectively.

3.2.5 Heterologous expression of CaNik1p and its mutated variants in *Saccharomyces cerevisiae*

S. cerevisiae transformed with pYES2 vector (Fig. 3.2) was selected and maintained on SD-ura, a synthetic medium deficient in uracil and containing glucose as a carbon source. The expression of the transgenes was controlled via the galactose promoter (*GAL1*). Therefore, to induce the transgene expression, the transformed strains were incubated in SG-ura, a synthetic medium deficient in uracil and containing galactose (to induce the *GAL1* promoter) and raffinose (to enhance the growth) as carbon sources.

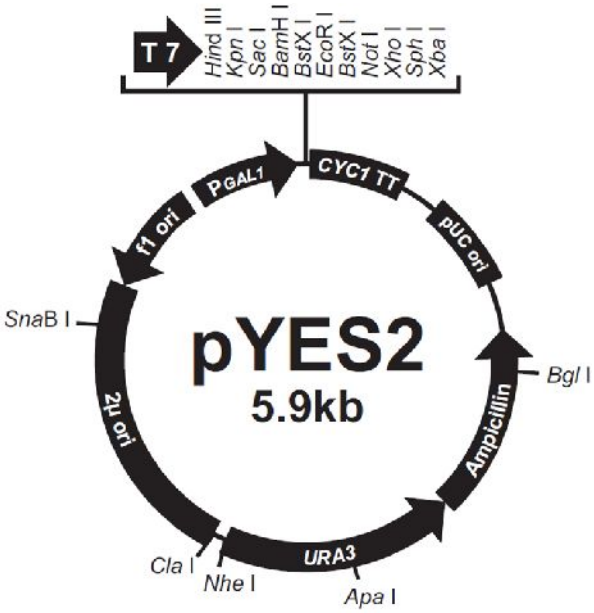


Fig. 3.2: Map of pYES2 vector (81).

Before induction of transgene expression, the transformants were cultivated at 30 °C in SD-ura for 36 h to obtain high cell densities. The cells were then pelleted and washed with SG-ura. To induce transgene expression, subsequent cultures were prepared in SG-ura as follows: an overnight culture (starting OD_{620nm} = 0.3), then a preculture (starting OD_{620nm} = 0.3, 2-3 h), and ultimately a working culture. The starting OD_{620nm} of the working culture and its cultivation period differed according to the purpose of the experiment (Tab. 3.7). For cultivation of the reference strains of *S. cerevisiae*, uracil was added at a final concentration of 40 mg/l to either SD-ura or SG-ura.

Purpose of experiment	Working culture	
	Starting OD_{620nm}	Cultivation period
Detection of phosphorylation of Hog1p in transformants of *S. cerevisiae*	0.3	15 min
Sensitivity of the transformants to various antifungals*	0.03	24 h
Production of CaNik1p and its mutated variants **	0.3	6 h

Tab. 3.7: Starting OD_{620nm} and incubation period of the working cultures used for heterologous expression of CaNik1p and its mutated variants in *S. cerevisiae* depending on the purpose of the experiment. (*: performed in 96-well microtiter plates, **: performed in 1 liter baffled flasks (5 flasks each containing 200 ml culture) with no preculture being prepared).

3.2.6 Cultivation of *C. albicans* strains

C. albicans strains were cultivated in YPD medium at 30 °C. To select and maintain strains transformed with the *SAT1* flipper cassette, NST was added to the medium at a final concentration of 200 µg/ml.

3.2.7 Protein methods

3.2.7.1 Protein extraction

3.2.7.1.1 Mikro-dismembrator (small scale)

Samples containing 10 ml cultures were centrifuged at 5000 rpm and RT for 4 min. The supernatant was discarded, and the cell pellet was resuspended in the residual medium. The cell suspension was dropped into a 50 ml falcon tube full of liquid N_2 to form cell pearls that could be stored at -30 °C until further disruption. The resulting cell pearls were then transferred into a 5 ml shaking flask (made of PTFE), which was pre-cooled in the liquid N_2 and contained a grinding ball. The shaking flask was immersed in liquid nitrogen for a few seconds, before disruption of the cell pearls by the shaking of the flask at 2000 rpm for 2 min in the Mikro-dismembrator. The resulting powder was transferred quickly into a 2 ml pre-cooled tube and allowed to thaw on ice. After this thawing step, the suspension was centrifuged at 8000 rpm and 4 °C and for 5 min. Some of the supernatant (15 µl) was kept for the determination of protein concentration as in 3.2.7.2.2, whereas the remainder was mixed

with an equal volume of lysis buffer A and shock-frozen in liquid N_2 before being stored at -80 °C.

3.2.7.1.2 Grinding by mortar (large scale, for purification of the heterologously expressed CaNik1p)

1 liter of the culture was centrifuged at 5000 rpm and RT for 4 min. The resulting cell pellets were collected in one falcon tube (50 ml) and washed with 10 ml washing buffer. The washed cell pellets were suspended in 10 ml lysis buffer B, which was then dropped into 50 ml falcon tube full of liquid N_2 to form cell pearls. The resulting cell pearls were stored at -80 °C. For cell disruption, the cell pearls were poured into a mortar that had previously been pre-cooled by the addition of liquid N_2 and were gently ground with the pre-cooled pestle. During grinding, liquid N_2 was added from time to time to keep the disrupted cells in the frozen state. Grinding was continued until a fine homogeneous white powder of the disrupted cells was obtained. The resulting powder was transferred into a pre-cooled 50 ml falcon tube and left on ice for 2 h until a homogeneous suspension of the disrupted cells was obtained without any frozen clumps.

3.2.7.2 Protein quantification

3.2.7.2.1 BCA method

The bicinchoninic acid (BCA) method (82) was used for determination of the protein concentration in total protein extracts obtained from *S. cerevisiae* strains. The principle of the BCA assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by the reduction of the Cu^{2+} to Cu^{1+}. The amount of reduced Cu^{1+} is proportional to the amount protein present. Cysteine, cystine, tryptophan, tyrosine, and peptide bonds have been shown to be able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with Cu^{1+} in alkaline environments (Fig. 3.3), thus providing a basis for monitoring the reduction of Cu^{2+} by proteins at an absorbance of 570 nm. Lysozyme was used as a standard protein and was prepared in a stock solution of 10 mg/ml in water, from which serial dilutions were further prepared.

In a clean reservoir, BCA-Reagent A and B were mixed at a ratio of 50:1 to form the assay solution. The assay was performed in 96-well microtiter plates in which 5 µl from the protein samples and various dilutions of lysozyme were applied as duplicates in different wells. Either lysis buffer A or B was used as negative control. The assay solution (195 µl) was applied to all samples. The plate was gently shaken for a few seconds before incubation at 37

°C for 30 min. The absorbance was then measured at 570 nm, and the protein concentrations were determined from standard curves of lysozyme after subtraction of the background of the control.

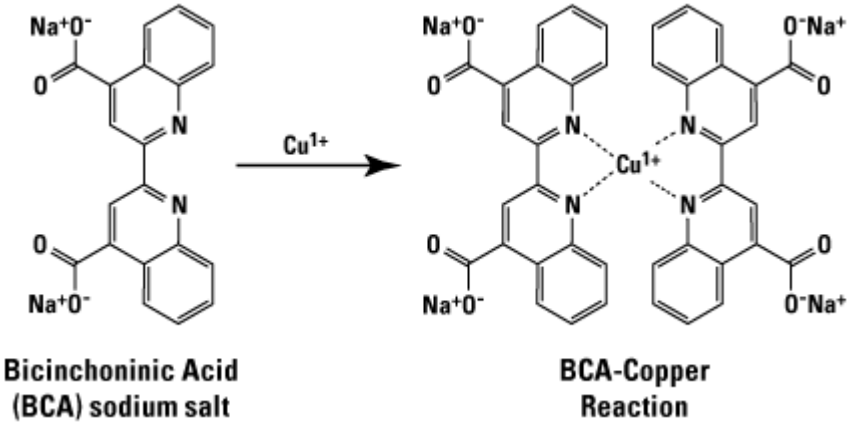


Fig. 3.3: The principle of the BCA reaction. Two molecules of BCA bind to each molecule of copper that had been reduced by a peptide-mediated reaction.

3.2.7.2.2 NanoDrop spectrophotometer

The concentration of purified CaNik1p and its mutated variants was determined by measuring the absorbance at 280 nm by using the NanoDrop 1000 spectrophotometer. The mass extinction coefficient of the protein was determined as E1% = 4.7 (83).

3.2.7.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein extracts were denatured by being heated at 95 °C for 10 min in the presence of the anionic detergent SDS and the reducing agent β-mercaptoethanol. In this step, the proteins are complexed by SDS and become negatively charged, thereby disrupting their subunits. During electrophoresis, the proteins were separated according to their molecular weight while migrating toward the anode (84). The protein samples were heated at 95 °C with 5x Laemmli buffer for 10 min before application into the wells of the stacking gel, and the gel was allowed to run at 80 V and 500 mA for 30 min and then at 120 V and 500 mA until the dye front had reached the end of the separating gel. To estimate the molecular weight of the separated proteins, the protein marker Precision Plus Protein Dual Color Standard (Bio-Rad) was loaded in parallel with the samples. Electrophoresis was carried out in a Mini Protein II Electrophoresis System and was run in 1x TGS buffer (SDS-PAGE running buffer). For routine SDS-PAGE, a 12.5 % separating gel was prepared, whereas for the separation of CaNik1p and its mutated variants, a 7.5 % separating gel was used.

3.2.7.4 Western blot

The separated proteins were transferred from SDS-PAGE gel onto a PVDF membrane by semi-dry transfer in a blot buffer. The transferred proteins were detected by the corresponding antibodies (Tab. 3.8).

A PVDF membrane and 2 filter papers (3 mm, Whatman) were cut according to the size of the gel (0.5 mm excess in width and length) and the membrane was moistened with methanol followed by washing with water. The membrane and the filter papers were incubated in the blot buffer till the transfer was started. After electrophoresis, the gel was incubated in the blot buffer for 30 min.

To start the transfer, the filter paper was placed toward the anode side of the Trans-Blot Semi-Dry Electrophoretic Transfer Cell, followed by the membrane which was completely covered with blot buffer before adding the gel and finally the other filter paper. The transfer was performed at 250 mA and 15 V for 30 min. The membrane was washed with TBS-T buffer for 5 min before being blocked by incubation with 5 % milk powder in TBS-T buffer at RT for 1 h. The membrane was washed with TBS-T buffer for 4 x 10 min.

For detection of phosphorylated Hog1p, the membrane was incubated at 4 ˚C with the P-p38 Ab (1:1000 in 5 % BSA in TBS-T) overnight. The membrane was then washed with TBS-T buffer for 4 x 10 min, incubated with the anti-rabbit Ab (1:6666 in 5 % BSA in TBS-T) at RT for 1 h followed by washing with TBS-T buffer for 4 x 10 min. The bound HRP-linked anti-rabbit Ab was visualized by detection of chemiluminescence that resulted from the addition a peroxidase–specific chemiluminescence substrate (ECL, advance Western blot detection kit) via the CCD camera of the LAS-3000. For detection of total Hog1p, the membrane was washed with TBS-T for 2 x 10 min, stripped by incubation in 1× Re-Blot Plus Solution (Millipore) for 15 min, and washed with TBS-T buffer for 2 x 10 min. The membrane was blocked with milk powder in TBS-T buffer and then incubated with Hog1 Ab (1:2000 in 5 % BSA in TBS-T) at RT for 2 h and detection was done as previously mentioned.

For the detection of CaNik1p and its mutated variants, the FLAG-tagged proteins were detected by incubating the blocked membrane with monoclonal anti-FLAG M2-Peroxidase Ab (1:50000 in 5% BSA in TBS-T) at 4 ˚C for 1 h. Blocking with milk powder, washing steps and visualization of the bound antibodies were carried out as previously mentioned.

Name	Clone	Epitope	Produced in	Company and Cat. No.
P-p38	mAb	Phospho-p38 MAPK (Thr180/Tyr182)	Rabbit	Cell Signaling, 9215
Hog1	pAb	Hog1 (y-215)	Rabbit	Santa Cruz, 9075
Anti-rabbit*	mAb	Rabbit IgG	Goat	CellSignalling, 7074
Anti-FLAGM2-Peroxidase*	mAb	FLAG tag (DYKDDDDK)	Mouse cell culture	Sigma Aldrich, A8592

Tab. 3.8: Antibodies used for Western blots (* Ab is conjugated to HRP).

3.2.8 CaNik1p purification from the cell lysate

The suspension with the disrupted cells obtained in 3.2.7.1.2 was centrifuged at 8000 rpm 4 °C for 5 min. Ni^{+2}-agarose beads (MachereyNagel) were used to capture the His-tagged CaNik1p and its mutated variants from the cell lysate, 2 ml agarose beads were sufficient to bind all the tagged protein in the cell lysate resulting form 500 ml working culture (OD_{620nm} 0.6-0.8). The beads were equilibrated with NPI-10 buffer as mentioned in the manufacturer's protocol before incubation with the cell lysate in 50 ml falcon tube, which was immersed in ice and shaken gently for 1 h. The unbound proteins were removed by centrifugation at 500 g and 4 °C for 10 min with subsequent rejection of the supernatant. To remove the non-specifically bound proteins, the beads were washed for 6 x 10 min with NPI-50 buffer (10x volumes of beads) by centrifugation at 500 g and 4 °C. The bound proteins were eluted by gentle shaking of the beads with of NPI-500 buffer (1x volume of beads) for 2 min while immersed in ice, followed by centrifugation at 500 g and 4 °C for 5 min. The elution step was repeated three times, and the supernatants (containing the eluted proteins) were pooled. To remove any residual beads, the eluate was passed through a Steriflip-NY Filter. The purified protein solution, which contained imidazole (500 mM), was then concentrated by centrifugation at 2000 g and 4 °C by using a 50 kDa MWCO Viva 20 spin column. The concentrated protein solution was ten-fold diluted with washing buffer and then concentrated again by ultrafiltration. The dilution process was repeated until the concentration of imidazole

reached 0.005 mM. The concentration of the purified protein was determined as in 3.2.7.2.2 before being shock-frozen in liquid N_2 and stored at -80 °C.

3.2.9 Protein identification by MALDI TOF-MS

After electrophoresis, protein bands of interest were excised from the gel by a clean scalpel, washed three times with deionized water, and then left to dry at 37 °C for 15 min. The dried protein bands were sent to the Cellular Proteome Research unit, HZI for further identification of the protein by MALDI TOF-MS.

3.2.10 Detection of phosphorylated peptides by LC-MS/MS

(Performed by Dr. Josef Wissing and Dr. Manfred Nimtz, Cellular Proteome Research unit, HZI)

Proteins were extracted with chloroform/methanol by using the method of Wessel and Flügge (85). For tryptic digestion, the extracted proteins were resolubilized in 50 mM TEAB (triethylammonium bicarbonate), reduced for 1 h at 56 °C with TCEP (triscarboxyethyl phosphine) and alkylated with MMTS (methylmethane thiosulfonate) for 20 min. Reduced and alkylated proteins were digested with trypsin at a final ratio of 50:1 (protein : protease) and incubated at 37 °C overnight. The digest was dried in a SpeedVac centrifuge. After evaporation of all the liquid in the SpeedVac, the peptides were desalted onto Zip Tip's. Peptides were resolubilized in 3 % ACN containing 0.2 % TFA and adsorbed to RP18 material. After washing with the binding solution, the peptides were eluted with 60 % ACN containing 0.2 % TFA. To remove the organic phase, the peptides were dried in a SpeedVac, resolubilized in 12 µl 3 % ACN containing 0.2 % TFA and centrifuged in an ultracentrifuge. Peptides were used then ready for LC-MS/MS analysis.

LC-MS/MS analysis was performed on a DionexUltiMate 3000 n-RSLC system connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Peptides were loaded onto a C18 pre-column (3 µm, Acclaim, 75 µm x 20 mm, Dionex) washed for 3 min at a flow rate of 6 µl/min. Subsequently, peptides were separated on a C_{18} analytical column (3-µm, Acclaim PepMap RSLC, 75 µmx 25 cm, Dionex) at 350 µl/min via a linear 30-min gradient from 100 % solution A (0.1 % formic acid in water) to 25 % solution B (99.9 % acetonitrile with 0.1 % formic acid) followed by a 15 min gradient from 25 % solution A to 80 % solution B. The LC system was operated by Chromeleon Software, which was embedded in Xcalibur software. The effluent from the column was electro-sprayed (Pico Tip Emitter Needles, New Objectives) into the mass spectrometer. The mass spectrometer was controlled by Xcalibur

software and operated in the data-dependent mode allowing the automatic selection of a maximum of 5 double- and triple-charged peptides and their subsequent fragmentation. A dynamic exclusion allowed up to 3 repeats. Peptide fragmentation was carried out by using LTQ settings (min signal 2000, isolation width 4, normalized collision energy 35, default Charge State 4, and activation time 10 ms). MS/MS raw data files were processed via Mascot Daemon-aided searching against the UniProtKB/Swiss-Prot protein database or by using the Proteome Discoverer program. The following search parameters were used: enzyme, trypsin; maximum missed cleavages, 1; fixed modification: carbamidomethylation (C); variable modifications: oxidation (M), phosphorylation of S, T, and Y, respectively; peptide tolerance, 5 ppm; MS/MS tolerance, 0.4 Da.

3.2.11 Investigation of the interaction between fludioxonil and purified CaNik1p via Saturation Transfer Difference-NMR (STD-NMR)

(Performed by Prof. Dr. Christiane Ritter, Macromolecular Interactions unit, HZI)

The possibility of the interaction between fludioxonil and CaNik1p was investigated via STD-NMR. The NMR spectrum of a saturated solution of fludioxonil in deuterated DMSO was recorded, after the addition of various concentrations of purified CaNik1p by using a BrukerAvance III spectrometer operating at a proton Larmor frequency of 600.28 MHz and equipped with a triple resonance cryoprobe platform. The final concentration of fludioxonil in the mixture was 100 µM. As control, fludioxonil was incubated with the flow-through of the ultrafiltration step in the purification protocol of CaNik1p (3.2.8).

3.2.12 *In vitro* kinase assay of the purified CaNik1p and its mutated variants

3.2.12.1 Kinase-Glo Plus kit

The kinase activity of purified CaNik1p and its mutated variants was determined by the Kinase-Glo Plus kit in white opaque 96-well microtiter plates. The assay is based on the detection of the ATP consumption during a kinase reaction (Fig. 3.4). ATP is quantified via the luciferin-luciferase reaction. The higher the kinase activity (or ATPase activity), the lower the final luminescence value as more ATP is consumed in the kinase reaction.

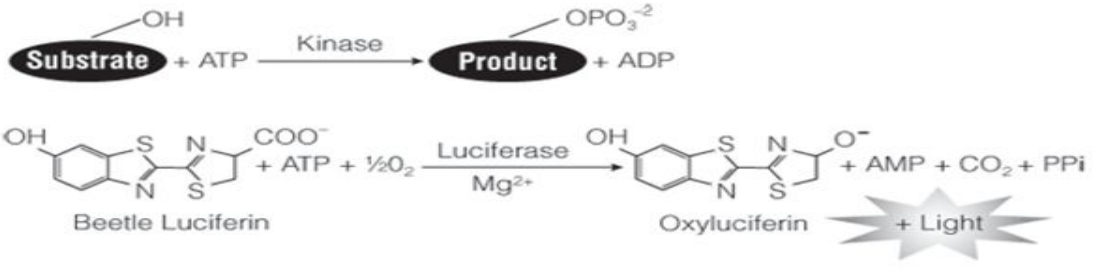


Fig. 3.4: Principle of the Kinase-Glo plus Kit (86).

The kinase reaction mixture (50 µl) (Tab. 3.9) comprised ATP and the purified protein. No additional substrate was required, as CaNik1p undergoes autophosphorylation. The mixture was incubated in the wells of microtiter plate at 30 °C for 2:30 h. The Kinase-Glo reagent (50 µl) was added and the plate was sealed with aluminum foil and incubated at 30 °C for 20 min. The plate was transferred to a luminescence microtiter plate reader (Synergy, Biotek) and after additional 10 min the luminescence was measured. The flow-through buffer (3.1.1.3) was used as negative control in the same plate. The control buffer contained the same imidazole concentration (0.005 mM) as all protein samples, which were obtained at the end of the purification procedure (3.2.8). The influence of fludioxonil on the kinase activity of purified CaNik1p was studied by addition of 40 µM (10 µg/ml) fludioxonil (5 % volume of the total kinase reaction) to the reaction mixture. The solvent of fludioxonil, DMSO, was used as a negative control in a separate sample.

Component	**Volume added to the kinase reaction (µl)**
ATP (100 µM) in 1x kinase buffer A	10 (final concentration 20 µM)
Protein sample	X
10x kinase buffer A	4
Water	50- (14+X)

Tab. 3.9: Components of the kinase reaction mixture when the kinase-Glo Plus kit was used for the *in vitro* kinase assay of CaNik1p and its mutated variants.

3.2.12.2 Incubation with the radiolabeled [γ-^{32}P] ATP

The purified protein (0.5 µM) was incubated in kinase buffer B containing hot ATP (1 µCi) and 110 µM ATP (cold ATP) in a final volume of 10 µl at 30 ˚C for 1 h. Denatured protein (preheated at 95 ˚C for 10 min) was used as a negative control in a separate kinase reaction. After incubation, the reaction was stopped by the addition of LDS buffer (NP0007, Invitrogen) and β mercaptoethanol (final concentration of 2.5 %) followed by heating at 95 ˚C for 10 min. The denatured proteins were loaded on NuPAGENovex 10 % Bis-Tris precasting gels (NP0301PK2, Invitrogen). Proteins were separated via gel electrophoresis at 150 mV for 1 h in the presence of NuPAGE MES SDS (NP0002, Invitrogen) as running buffer. To estimate the molecular weight of the separated proteins, the protein marker Precision Plus Protein Dual Color Standard was used. The protein bands were visualized after electrophoresis by staining with InstantBlue (Expedeon) for 1 h. The gel was exposed to a phosphor screen at RT for 4 h after being washed with PBS overnight to minimize background resulting from unreacted hot ATP. The phosphor screen was scanned via the phosphorimage analyzer BAS2500. The influence of fludioxonil on the autophosphorylation of CaNik1p was investigated at a final concentration of 40 µM (10 µg/ml). The solvent of fludioxonil, DMSO, was used as a negative control.

3.2.13 Molecular biology methods

3.2.13.1 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from 4 ml overnight bacterial cultures by using the Miniprep Qiagen plasmid DNA Extraction kit according to the manufacturer's instructions.

3.2.13.2 Isolation of genomic DNA from *C. albicans* (74)

Cells from 10 ml overnight culture were pelleted and washed with deionized water before being suspended in 200 µl breaking buffer (3.1.1.4). 200 µl glass bead (0.25-0.5 mm, Roth) and 200 µl phenol/chloroform/isoamyl alchohol mixture (25:24:1, Roth) were added to the cell suspension, and the cells were broken by vortexing for 5 min. TE buffer (200 µl) was then added, and the suspension was mixed for 45 sec by gentle inverting before being centrifuged at 10,000 rpm for 5 min. The upper aqueous layer was carefully transferred into a 2 ml tube. For precipitation of the DNA, a 2.3 volume of 100 % ethanol was added, and the solution was mixed by inversion before centrifugation at 10,000 rpm for 2 min. The supernatant was carefully rejected, and the precipitated DNA pellet was left to dry at 37 °C till complete evaporation of any residual ethanol. The pelleted DNA was dissolved in 100 µl

10 mM Tris-HCl (pH 7.5) containing RNaseA (10 µg/ml). The DNA concentration was determined as in 3.2.13.6 and stored at -20 °C.

3.2.13.3 Amplification of DNA by Polymerase Chain Reaction (PCR)

DNA Polymerases DreamTaq (Fermentas) and Phusion High-Fidelity (New England Biolabs) were used for routine PCR and cloning purposes respectively. The conditions of the PCR and the various concentrations of components were adjusted according to the manufacturer's protocol for each DNA polymerase. After adjusting the annealing temperature of the primers, the thermocycler was programmed to run the PCR cycles.

3.2.13.4 Agarose gel electrophoresis

The DNA fragments were separated according to their size by electrophoresis in 0.5-1 % agarose gels. The required amount of agarose was dissolved in 1x TAE buffer by heating the mixture in a microwave oven. Ethidium bromide solution was added to the agarose solution and mixed well. This solution was poured into a gel tray. The DNA samples were mixed with 6x loading buffer (New England Biolabs) and loaded onto the solidified gel. Electrophoresis was performed at 100 V in 1x TAE buffer. To estimate the size of nucleic acids, a 2-Log DNA Ladder (New England Biolab) was run parallel to the samples in the same gel. A combined system of UV-Transilluminator and gel documentation was used to visualize and photograph the fluorescence of the DNA-bound ethidium bromide.

3.2.13.5 Purification of DNA from agarose gel

The DNA fragments of interest were excised from the gel by a clean scalpel and further purified via the QIAquick Gel Extraction Kit according to the manufacturer's protocol.

3.2.13.6 Measurement of DNA concentration

The concentration of the DNA was measured via the NanoDrop 1000 spectrophotometer by measuring the absorbance at 260 nm. The purity of the DNA was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7 - 1.9.

3.2.13.7 Sequencing of inserts and constructs

All vector constructs were confirmed by DNA sequencing in order to ensure correct insertions or mutations. After isolation of the plasmid (30 µl, 250-500 ng/µl), it was sent together with primers (5 µl, 10 pmol) spanning the target sequences either to the Eurofins company (Ebersberg, Germany) or the GMAK sequencing service unit in HZI

(Braunschweig, Germany) for sequencing. Tab. 3.4B shows the various primers that were used for the sequencing of the different constructs.

3.2.13.8 Transformation of chemically competent *E. coli* by heat shock method

An aliquot (50 µl) of chemically competent *E. coli* cells (Stellar or XL1-Blue) was transformed with the desired plasmid by heat shock. The transformation and further cultivation procedures were performed according to the manufacturer's instructions.

3.2.13.9 "Quick and dirty" transformation of *S. cerevisiae*

The "Quick and dirty" method (80) was used for the transformation of the reference strains of *S. cerevisiae* BWG1-7A or BY4741 with the pYES2 vector harboring the mutated variants of *CaNIK1*.

In a sterile 2 ml tube, 3 µl denatured salmon sperm DNA, 5 µl plasmid DNA (1 µg), and 100 µl yeast transformation mixture (3.1.1.4) were mixed together. A large yeast colony of freshly growing *S. cerevisiae* was picked from a YDD agar plate and suspended in the previous mixture. The cell suspension was incubated for 30 min at 37 °C with shaking at 160 rpm. The suspension was centrifuged for 5 min at 3000 rpm, and the resulting pellet was suspended in 100 µl sterile water before being streaked on an SD-ura agar plate. The plate was incubated for 3-4 days at 30 °C. The resulting colonies, which represented transformed cells, were cultivated in SD-ura and then processed as in 3.2.1.

3.2.13.10 Transformation of *C. albicans* by electroporation

YPD (20 ml) was inoculated with 300 µl cryo-stock culture of *C. albicans* Sc5314 and incubated overnight at 30 °C with shaking at 160 rpm. A working culture (50 ml) was prepared from the overnight culture with a starting OD_{620nm} = 0.08 and was incubated at 30 °C with shaking at 160 rpm. After 2:30 h, the culture (OD_{620nm} = 0.2) was transferred into a sterile 50 ml falcon tube and centrifuged for 5 min at 5000 rpm. The supernatant was discarded, and the cell pellet was suspended in 8 ml sterile deionized H_2O. One ml of both 10× TE buffer (pH 7.5) and 1 M lithium acetate (pH 7.5) was added, and the tube was inverted gently for proper mixing before incubation at 30 °C with shaking at 160 rpm for 50 min. 250 µl 1 M dithiothreitol (DTT) was then added, and the tube was further incubated at 30 °C for 25 min with shaking at 160 rpm. Sterile cold deionized H_2O (40 ml) was added, and the cells were centrifuged for 5 min at 5000 rpm and 4 °C.

From this step until the end of the transformation process, the cells were always kept on ice or at 4 °C. After centrifugation, the supernatant was discarded, and the cells were

suspended in 25 ml sterile cold deionized H_2O and centrifuged for 5 min at 5000 rpm and 4 °C (5804R, Eppendorf). After centrifugation, the supernatant was rejected, and the cells were suspended in 1 ml sterile cold 1 M sorbitol. The suspension of cells was transferred into 2 ml tubes (pre-cooled on ice) and centrifuged for 5 min at 5000 rpm and 4 °C. The supernatant was discarded, and the cells were resuspended in the remaining liquid to obtain a dense suspension (ca. 200 µl). The cell suspension (40 µl) was mixed with 5 µl of the purified DNA fragment (at least 1 µg) that harbored the deletion cassette in a pre-cooled 2 ml tube; mock cells (cells without DNA) were also prepared. The tubes were then immersed in ice for 5 min before the cells were transferred into a pre-cooled electroporation cuvette (0.2 mm) that was then placed into an Equibioelectroporator at 1.5 kV, 25 µF, 200 Ω. After electroporation, the cuvette was placed back on ice, and immediately 1 ml YPD medium was added. The cells were suspended by pipetting up and down. The cell suspension was then transferred into a 2 ml tube, which was incubated at 30 °C for 5 h with shaking at 160 rpm. 100 µl cell suspension was then streaked onto a YPD agar plate containing 200 µg/ml NST. As a control, 100 µl 10^{-4} and 10^{-5} diluted cell suspension was streaked onto YPD agar plates without NST in order to determine the number of viable cells. The remaining cell suspension was centrifuged at 5000 rpm for 2 min, and the supernatant was discarded. The cells were resuspended in the remaining liquid and streaked onto a YPD agar plate containing 200 µg/ml NST. The plates were incubated for 2 days at 30 °C, and the resulting colonies were analyzed by colony PCR for the desired allelic replacement.

3.2.13.11 Restriction digestion of DNA fragments

The reaction conditions for digestion of DNA fragments with restriction enzymes (all purchased from New England Biolabs) were as recommended by the manufacturer.

3.2.13.12 Cloning of PCR products

For ligation of the purified DNA fragments, either T4 DNA ligase (New England Biolabs) or the In-Fusion HD Cloning Kit (Clontech) was used.

The T4 DNA ligase was used to ligate the restriction enzyme digest of pYES2 vector and the corresponding PCR product to yield the plasmid pYES2-*CaNIK1*ΔHAMPup.

The In-Fusion HD Cloning Kit (Fig. 3.5) enables directional cloning of one or more fragments of DNA into vectors. The cornerstone of the In-Fusion Cloning technology is the In-Fusion enzyme, which fuses DNA fragments e.g. PCR-generated sequences and linearized vectors, efficiently and precisely by recognizing 15 bp overlapping sequences at their ends.

This 15 bp overlap can be engineered by designing primers for the amplification of the desired sequences. The guidelines of the manufacturer for the design of the primers were followed to ensure that PCR products will be obtained containing ends that were homologous to those of the linearized vector. The In-Fusion enzyme was used to delete the sequence encoding all the HAMP domains in *CaNIK1* (yielding the plasmid pYES2-*CaNIK1*ΔHAMP). In addition, it was used to construct the *CaNIK1* deletion cassette in the pNIK plasmid.

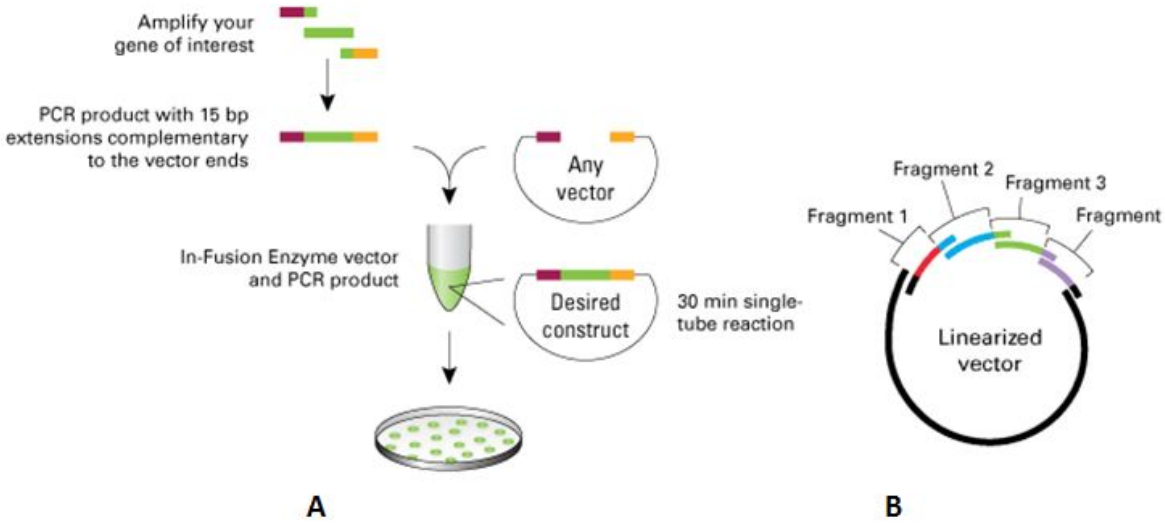


Fig. 3.5: The In-Fusion HD Cloning system. A) Protocol B) Ability to ligate multiple fragments in a single reaction (87).

3.2.13.13 QuikChange site-directed mutagenesis kit as a tool to introduce point mutations

The QuikChange site-directed mutagenesis kit is used to introduce point mutations and to delete or insert single or multiple amino acids. The mutagenesis is performed by using PfuTurbo DNA polymerase and a temperature cycler. It depends on the design of primers that contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by the DNA polymerase. The use of mutagenic primers generates a mutated plasmid containing staggered nicks. After temperature cycling, the product is treated with DpnI. The DpnI endonuclease (target sequence: 5´-Gm^{6}ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. Therefore, the DNA should be isolated from a dam^{+} *E. coli* strain to be susceptible for DpnI digestion. The nicked vector

DNA containing the desired mutations is then transformed into XL1-Blue supercompetent cells. The overall principle of the kit is illustrated in Fig. 3.6.

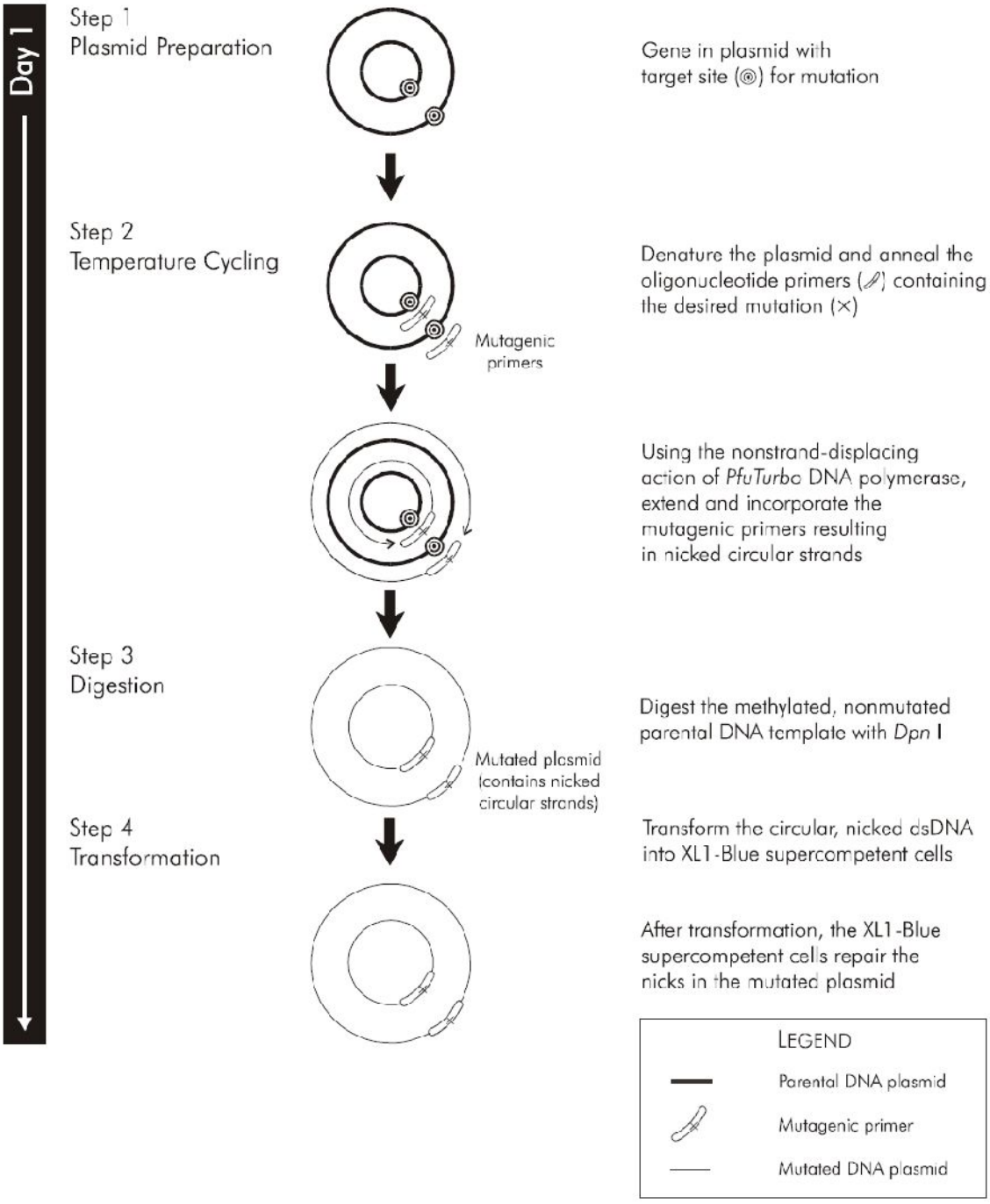


Fig. 3.6: Overview of the principle of the QuikChange site-directed mutagenesis kit (88).

3.2.14 Susceptibility testing of the transformed *S. cerevisiae* strains

The antifungal susceptibility test was carried out in sterile CytoOne 96-well plates as illustrated in Fig. 3.7. 135 µl of SG-ura was transferred into wells of the 2nd column, whereas

90 µl was added to wells of the 3rd to 11th column. In the 2nd column, 2 µl of the stock solution of the antifungal in methanol was added into three repetitive wells so that the concentration of the antifungals in the wells (135 µl SG-ura) was 2x the planed final concentration of the antifungal. To another three wells of the 2nd column, 2 µl of methanol without antifungal (as a control) was applied. The antifungal was serially diluted by transferring 45 µl from wells of the 2nd column to wells of the 3rd column, mixed thoroughly by pipetting up and down 5 times and repeating this step up to the 10th column. From the 10th column, the 45 µl was rejected so that the 11th column received neither antifungal nor methanol.

A preculture of the transformed strain in SG-ura was prepared (3.2.5). After 2-3 h incubation, the preculture was diluted with SG-ura to an OD_{620nm} of 0.06, from which 90 µl was added to all wells containing SG-ura medium, starting with the 11th column, to attain a final OD_{620nm} of 0.03. The plate was incubated at 30 °C with shaking at 500 rpm in a microtiter plate shaker (Titramax 1000). After 24 h incubation, the OD_{620nm} was measured and the IC50 was determined.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		3000	1000	333	111	37	12	4	1.3	0.47	No antifungal	
C		3000	1000	333	111	37	12	4	1.3	0.47	No antifungal	
D		3000	1000	333	111	37	12	4	1.3	0.47	No antifungal	
E		methanol									No methanol	
F		methanol									No methanol	
G		methanol									No methanol	
H												

Three-fold serial dilution

Fig. 3.7: Schematic diagram illustrating the three-fold serial dilution followed for IC50 determination of antifungals. : Antifungal concentration (ng/ml) (Initial concentration is for example 3000 ng/ml), : Control, methanol without antifungals.

4 Results

4.1 Detection of purified CaNik1p by Coomassie staining of SDS-PAGE and Western blot

The plasmid pYES2-*CaNIK1*-TAG (47) was used for the expression of the wild-type CaNik1p protein fused to a HIS/FLAG tag at the C-terminus (Fig. 4.1). The expressed dual-tagged CaNik1p was purified from the protein extract of strain NIK via the HIS-tag by using Ni^{+2}-agarose beads. The purification procedure was evaluated by separation with SDS-PAGE followed by Coomassie staining and the purified protein was detected by Western blot with anti-FLAG Ab (Fig. 4.2). The purified protein appeared at the expected molecular weight (121 kDa), and the band was excised and analyzed by MALD-TOF-MS. According to a Mascot data base search, the resulting peptide mass spectrum (Appendix II, page 98) was identified as CaNik1p.

Fig. 4.1: Scheme of the HIS-FLAG-tagged CaNik1p. (The HIS/FLAG tag is not drawn to scale).

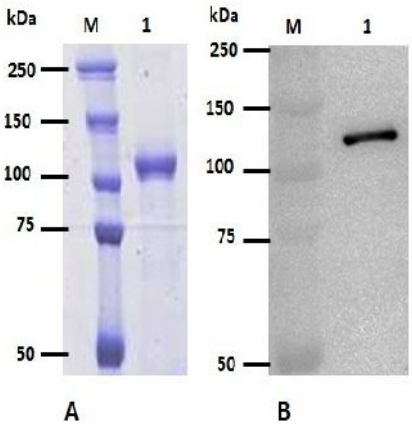


Fig. 4.2: Purified CaNik1p detected by Coomassie staining of SDS-PAGE and Western blot by using anti-FLAG Ab as indicated in A and B, respectively. M: Marker and 1: Purified CaNik1p.

4.2 Construction of various mutations in the *CaNIK1* gene

The plasmid pYES2-*CaNIK1*-TAG (47) was used as a template for all mutations of the *CaNIK1* generated in the present work. All mutations and the strategies utilized for their construction are illustrated in Fig. 4.3.

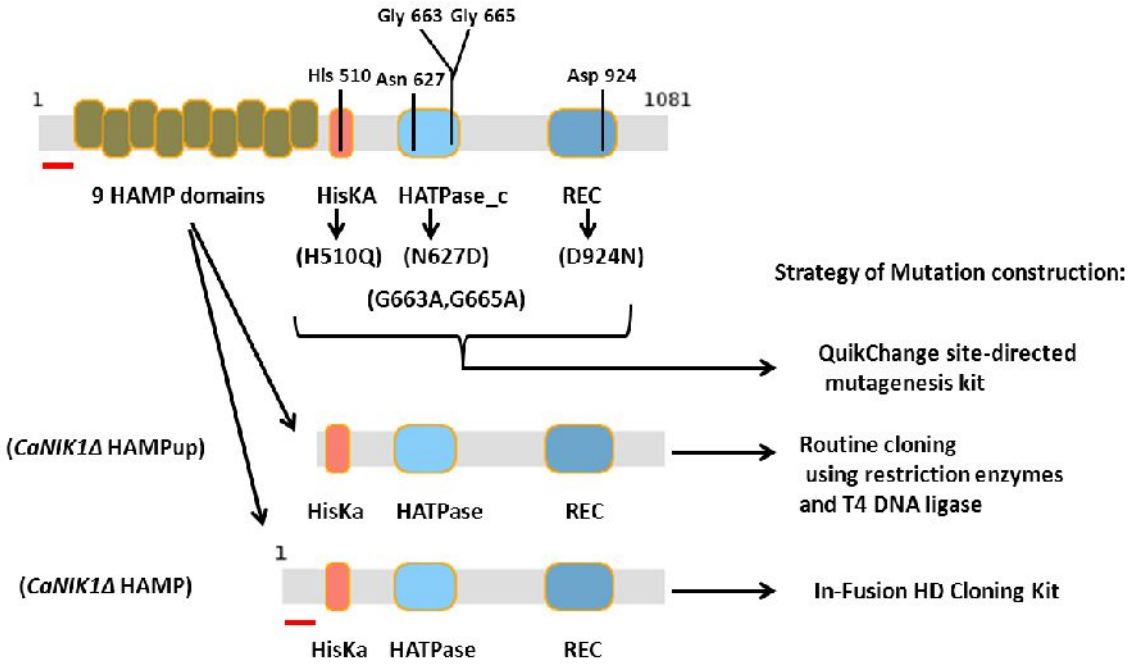


Fig. 4.3: Schematic representation of all the mutations of *CaNIK1* and the strategies used in their construction.

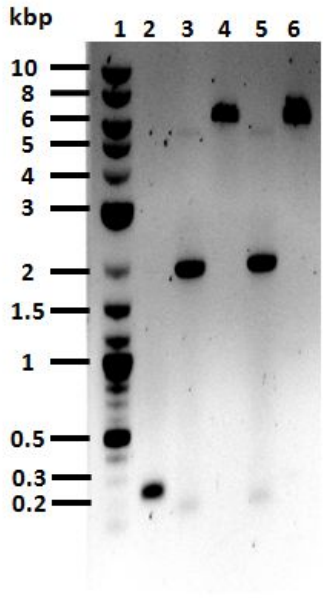
All point mutations in *CaNIK1* were performed by using the QuikChange site-directed Mutagenesis Kit (Strategene). Point mutations in the HisKA (H510Q), HATPase_c ((N627D) or (G663A, G665A)) and REC (D924N) domains were constructed by using the appropriate primers (Tab. 3.4). The design of mutagenic primers containing the desired mutations, PCR conditions, digestion with DpnI, and transformation in competent cells were carried out according to the manufacturer's instructions.

Two variants of CaNik1p, from which the HAMP domains were deleted, were constructed. The first (*CaNIK1*ΔHAMP) contained only a deletion of the sequence encoding HAMP domains (63-485 aa) in *CaNIK1*, whereas the second (*CaNIK1*ΔHAMPup) contained a deletion of the sequence encoding HAMP domains and its upstream part in *CaNIK1* (1-485 aa).

For construction of the plasmid pYES2-*CaNIK1*ΔHAMP, the pYES2 vector was linearized by using the restriction enzyme HindIII, and the pYES-*CaNIK1*-TAG plasmid was used as a

template for amplification of the following *CaNIK1* fragments. The sequence of *CaNIK1* upstream of the fragment encoding the HAMP domains (1-186 bp) was amplified by using the HMPF1 and HMPR1 primers (Tab. 3.4). HMPF1 included the homologous 15 bp stretch with the end of the linearized vector downstream the galactose promoter. The *CaNIK1* fragment (1454-3243 bp) located downstream the sequence encoding the HAMP domains and extended by the HIS-FLAG tag was amplified by using the HMPF2 and HMPR2 primers. HMPF2 and HMPR2 shared 15 bp homologous stretches with the 172-186 bp fragment of *CaNIK1* and with the other end of the HindIII-linearized pYES2 vector, respectively. HindIII restriction sites were introduced to the sequences of the HMPF1 and HMPR2 primers (Tab. 3.4). After separation of the PCR-amplified fragments by electrophoresis (Fig. 4.4), the gel pieces carrying the amplification products were excised, and the DNA was purified by using the QIAquick Gel Extraction kit. The purified fragments were ligated into the HindIII-digested pYES2 vector by using the In-Fusion enzyme according to the manufacturer's instructions to yield the plasmid pYES2-*CaNIK1*ΔHAMP.

For the construction of pYES2-*CaNIK1*ΔHAMPup, the *CaNIK1* fragment (1454-3243 bp) located downstream the sequence encoding the HAMP domains and extended by the HIS-FLAG tag was amplified from pYES-*CaNIK1*-TAG by using the forward primer HisKF2 and the reverse primer HisKR (Fig. 4.4). The primers HisKF2 and HisKR contained the HindIII and XbaI restriction sites respectively. The HindIII - XbaI-restricted PCR fragment was ligated to HindIII - XbaI-cleaved pYES2 (Fig. 4.4) to yield the plasmid pYES2-*CaNIK1*ΔHAMPup .



Lane	Sample	Expected size (bp)
1	Marker	-
2	PCR of pYES2-*CaNIK1*-TAG using HMPF1 and HMPR1	202
3	PCR of pYES2-*CaNIK1*-TAG using HMPF2 and HMPR2	1888
4	Digestion of pYES2 with HindIII	5856
5	PCR of pYES2-*CaNIK1*-TAG using HisKF2 and HisKR.	1884
6	Digestion of pYES2 with HindIII and XbaI	5762

Fig. 4.4: Various fragments used in the construction of pYES2-*CaNIK1*ΔHAMP and pYES2-*CaNIK1*ΔHAMPup separated by agarose gel electrophoresis.

4.3 Confirmation of the different constructed mutations of *CaNIK1*

4.3.1 Restriction enzyme digestion of the constructed plasmids

The integrity, correct gene insertions and insertion of mutations in the different plasmids (Fig. 4.5) were analyzed by restriction enzyme digestion. The resulting products were separated by gel electrophoresis (Fig. 4.6) and showed the expected bands. Final confirmation was achieved by sequencing.

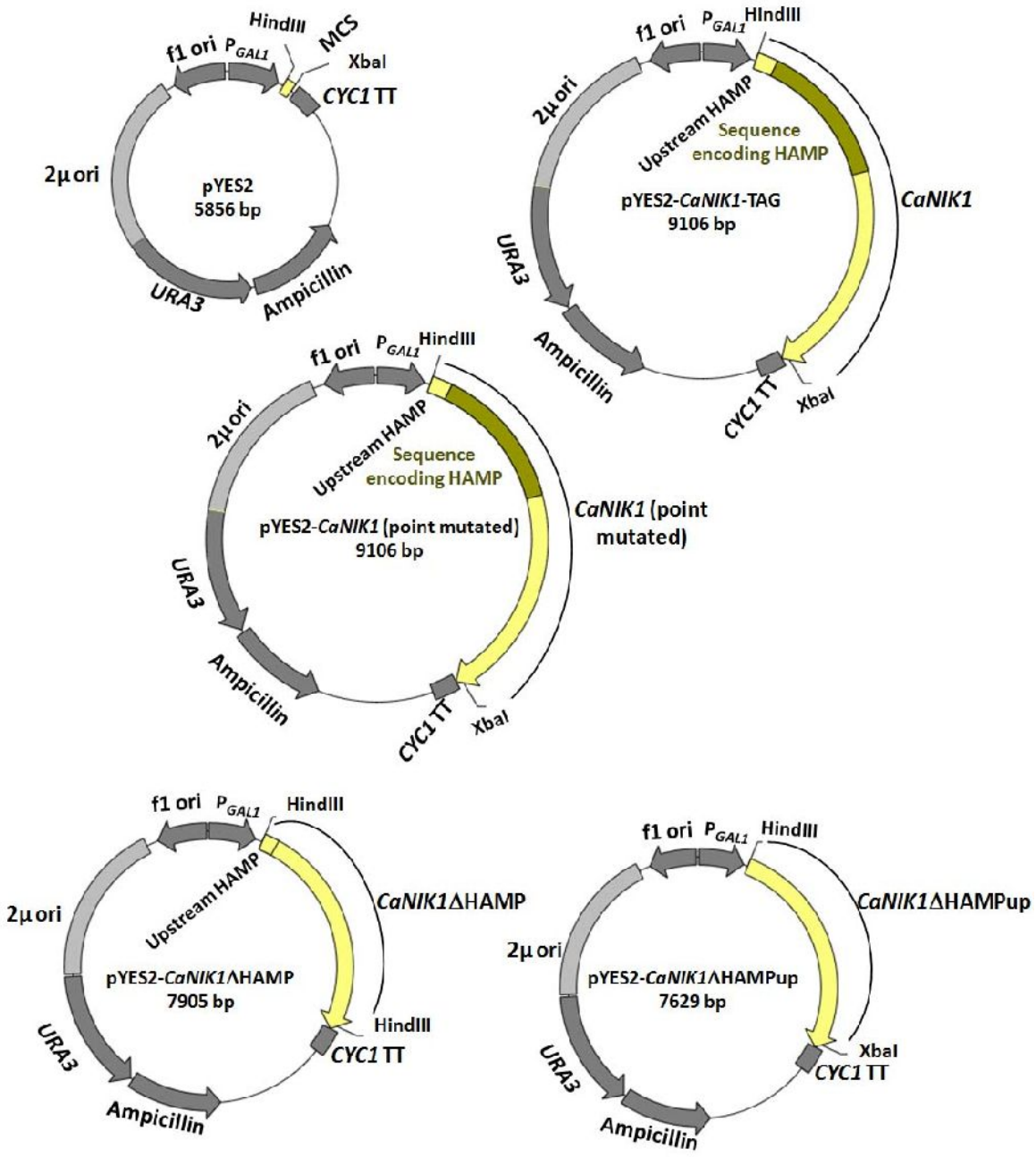
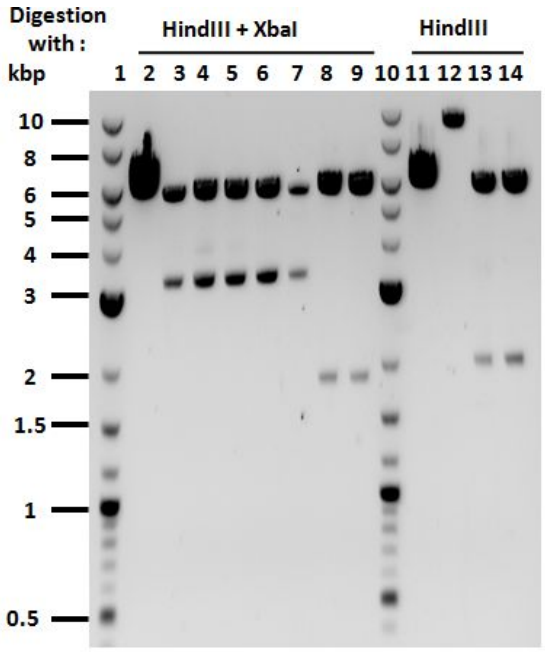


Fig. 4.5: Maps of the various plasmids used in the transformation of *S. cerevisiae*.

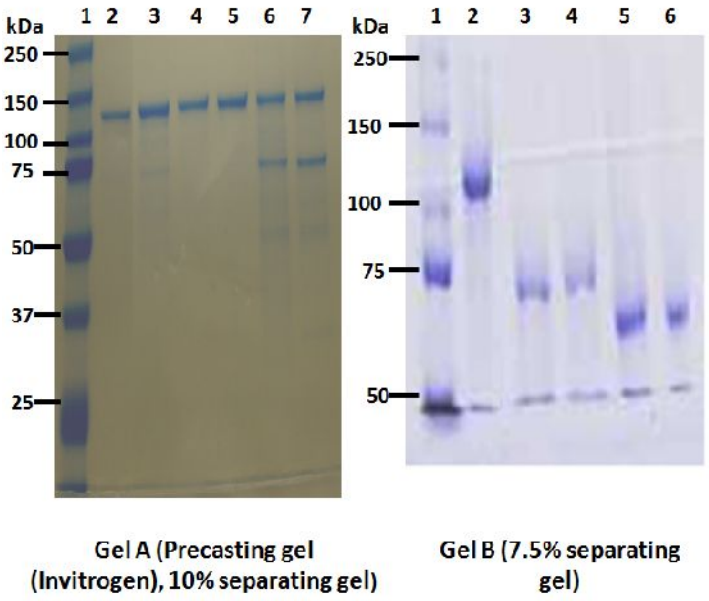


Lane	Sample	Expected size (bp)
1	Marker	-
2	pYES2	5763
3	pYES2-*CaNIK1*-TAG	5763 and 3343
4	pYES2-*CaNIK1*(H510Q)	
5	pYES2-*CaNIK1*(D924N)	
6	pYES2-*CaNIK1*(H510Q, D924N)	
7	pYES2-*CaNIK1*(N627D)	
8	pYES2-*CaNIK1*ΔHAMPup	1868 and 5761
9	pYES2-*CaNIK1*ΔHAMPup(H510Q)	1868 and 5761
10	Marker	-
11	pYES2	5856
12	pYES2-*CaNIK1*-TAG	9106
13	pYES2-*CaNIK1*ΔHAMP	2050 and 5855
14	pYES2-*CaNIK1*ΔHAMP(H510Q)	2050 and 5855

Fig. 4.6: Restriction digestion of the constructed plasmids containing different mutated variants of the *CaNIK1* gene.

4.3.2 Coomassie staining of SDS-PAGE of the expressed mutated variants of CaNik1p

To check for the successful expression and purity of the mutated variants of CaNik1p, samples resulting from protein purification procedures were separated by SDS-PAGE and stained with Coomassie (Fig. 4.7). All protein samples showed a single band at the expected MW except the samples containing protein with point mutations in the HATPase_c domain, namely CaNik1p(N627D) and CaNik1p(G663A, G665A). In the respective gels (lanes 6 and 7), two bands could be seen, of which the one with the higher molecular weight was the respective CaNik1p mutant. The smaller protein (MW 70 kDa) was identified as heat shock protein 71 (Hsp71), also called Ssa1, by MALDI-TOF-MS analysis (Appendix II, page 99).



Lane	Sample	Expected MW (kDa)
Gel A		
1	Marker	-
2	CaNik1p	121
3	CaNik1p(H510Q)	
4	CaNik1p(D924N)	
5	CaNik1p(H510Q, D924N)	
6	CaNik1p(N627D)	
7	CaNik1p(G663A, G665A)	
Gel B		
1	Marker	
2	CaNik1p	121
3	CaNik1pΔHAMP	75
4	CaNik1pΔHAMP (H510Q)	
5	CaNik1pΔHAMPup	68
6	CaNik1pΔHAMPup (H510Q)	

Fig. 4.7: Coomassie-stained SDS-PAGE of all the produced and purified mutated variants of CaNik1p.

4.4 Yield of the purified CaNik1p and its mutated variants after expression in various transformants

The expression and purification of CaNik1p and its mutated variants in various *S. cerevisiae* strains were carried out according to 3.2.5 and 3.2.8, respectively. The yields of different proteins obtained from the cell pellets of a 1 liter working cultures in SG-ura (OD_{620nm} 0.6-0.8) and after 5-6 h cultivation are presented in Tab. 4.1. It is obvious that expression of the protein variants in the wild-type strain BWG1-7a resulted in yields of less than 0.1mg/l working culture. Only from the protein being mutated in Asp924 (CaNik1p(D924N)) a significantly higher amount could be obtained (0.5 mg/l). We wondered whether the expression of a second HK in *S. cerevisiae* might be down regulated by the HOG pathway or the MAP kinase Hog1p and used the single gene deletion mutant Δhog of *S. cerevisiae* as a host for protein expression. Indeed, the yields of all proteins could significantly be increased, reaching almost 1 mg/l for the wild-type protein and the mutated protein CaNik1p(H510Q). Therefore, when high yields of purified proteins were required, e.g. for further *in vitro* kinase assays with the purified proteins, the deletion mutant Δhog was used as host for protein expression.

Yield (mg/l working culture) of various proteins expressed in *S. cerevisiae*		
Protein	Strain transformed with plasmid harboring the *CaNIK1* or one of its mutated variants	
	BWG1-7a	Δhog
CaNik1p	0.09	0.85
CaNik1p(H510Q)	0.052	1.02
CaNik1p(D924N)	0.54	ND
CaNik1p(N627D)	0.008	0.052
CaNik1p(G663A,G665A)	0.008	0.06
CaNik1p(H510Q, D924N)	ND	1
CaNik1pΔHAMP	ND	0.2
CaNik1pΔHAMP(H510Q)	0.13	ND
CaNik1pΔHAMPup	ND	0.24
CaNik1pΔHAMPup(H510Q)	0.06	ND

Tab. 4.1: The yield of various proteins expressed in various strains of *S. cerevisiae.* The yield values are the average of three independent experiments. (ND: Not done)

4.5 Susceptibility testing of the constructed mutants against various antifungals

We had previously shown that *S. cerevisiae* became susceptible to the selected antifungals, when CaNik1p was heterologously expressed (47). Thus we investigated the antifungal susceptibility of *S. cerevisiae* transformants, in which *CaNIK1* mutated variants were expressed.

The *S. cerevisiae* transformants were treated with various concentrations of the antifungals fludioxonil, iprodione and ambruticin VS3. As shown in Fig. 4.8, the strain (YES) transformed with the empty vector was resistant to all fungicides, whereas strain NIK was susceptible to the selected antifungals. The H510Q and D924N point mutations in the HisKA and REC domains, respectively, led to complete loss of susceptibility as indicated in strains H510 and D924. Although the N627D replacement in the HATPase_c domain (strain N627) decreased partially the fungicide susceptibility in comparison with the strain NIK, the double point mutations (G663A, G665A) were also associated with complete loss of susceptibility to the tested antifungals (strain G1). The IC50 values of the different antifungals are listed in Tab. 4.2.. The IC50 is defined as the concentration of the antimicrobial agent at which the growth of the culture is reduced to 50 % in comparison to the untreated culture.

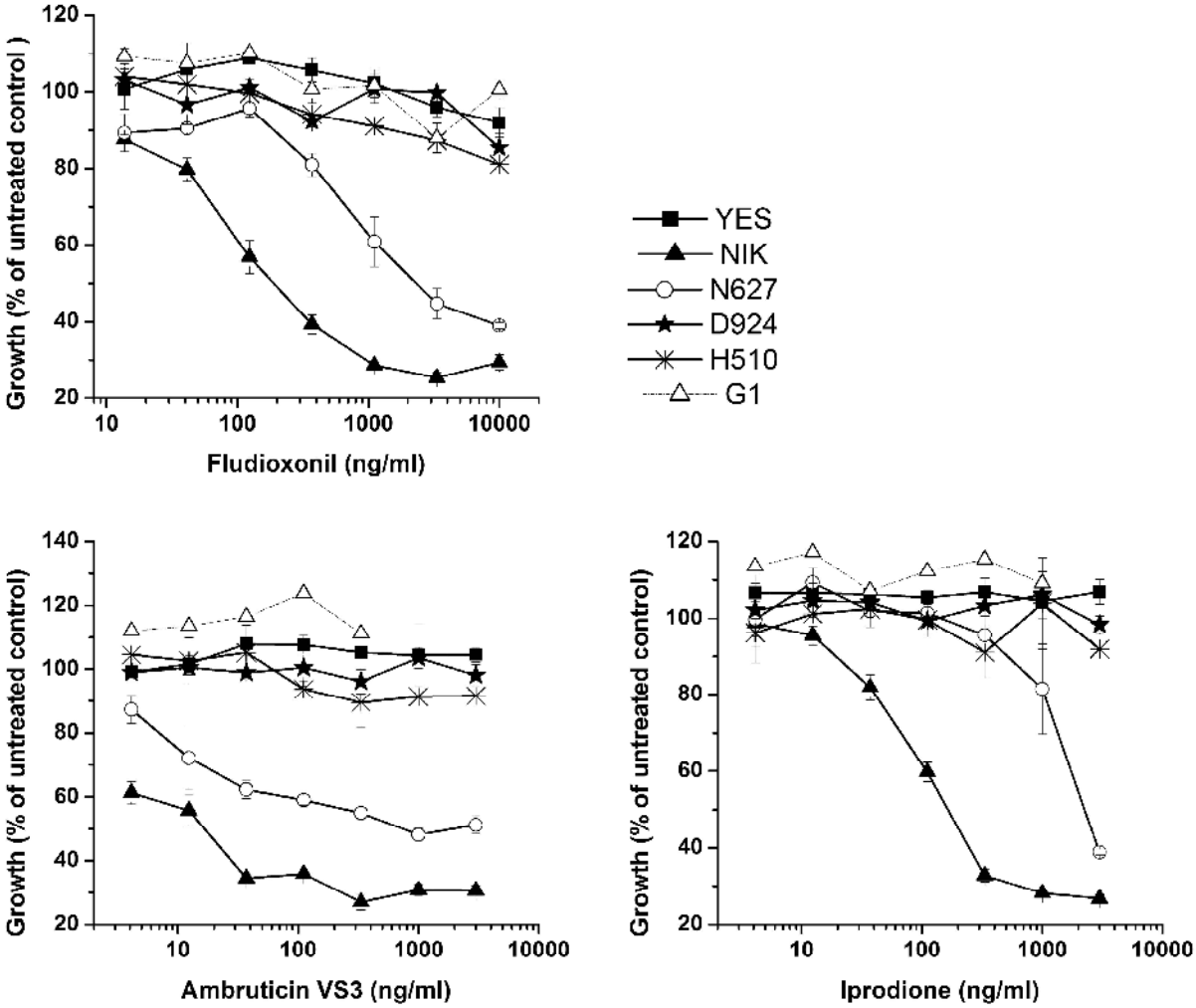


Fig. 4.8: Susceptibility of *S. cerevisiae* strains transformed with various mutants of *CaNIK1* to different antifungals.

Antifungal	IC50 (ng/ml) for *S. cerevisiae* transformed with	
	pYES2-*CaNIK1*-TAG	pYES2-*CaNIK1*(N627D)
Fludioxonil	220	2616
Ambruticin VS3	19	824
Iprodione	190	2478

Tab. 4.2: The IC50 (ng/ml) of the antifungals tested against various transformants of *S. cerevisiae*. The other transformants were totally resistant, even at the highest concentrations of the tested antifungals as shown in Fig. 4.8.

4.6 Functional HisKA, HATPase_c and REC domains are essential for the phosphorylation of the Hog1p after fludioxonil treatment

Treatment with fludioxonil led to phosphorylation of the MAPK Hog1p, i.e., to the activation of the Hog1 MAPK module, in *S. cerevisiae* transformed with full–length and

truncated forms of the *CaNIK1* gene (47). Therefore, phosphorylation of Hog1p was also analyzed after fludioxonil treatment (10 µg/ml for 15 min) of *S. cerevisiae* transformed with the *CaNIK1* gene carrying the H510Q, N627D and D924N point mutations. To establish the functionality of the HOG pathway in the tested transformants, sorbitol (1 M) was used as a positive control, since it is known to stimulate phosphorylation of the MAPK Hog1p via the induction of osmotic stress (89). The results (Fig. 4.9) demonstrated the complete abolishment of the phosphorylation of Hog1p in *S. cerevisiae* transformants carrying the D924N and the H510Q point mutations of the *CaNIK1* gene, whereas only a partial loss of the phosphorylation was observed in the transformant having the N627D substitution. Treatment of the transformants with 1 M sorbitol was associated with the phosphorylation of Hog1p thereby indicating the functionality of the HOG pathway in these transformants.

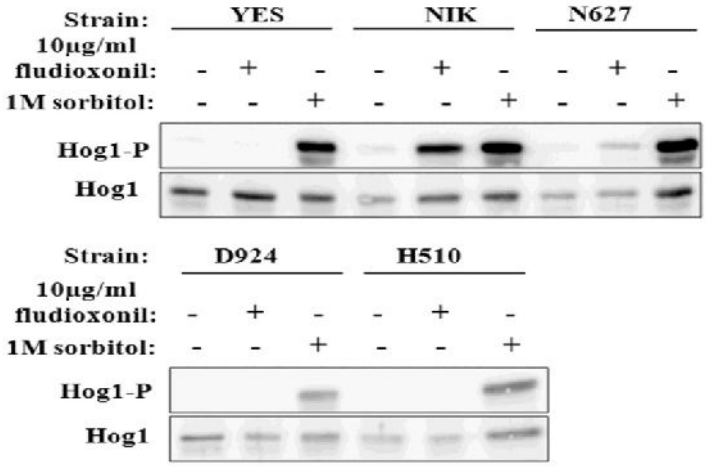


Fig. 4.9: Detection of phosphorylation of Hog1p by Western blot after treatment of various transformants with fludioxonil. Phosphorylation of Hog1p (upper panel, Hog1-P) is examined after treatment of strains YES, NIK, N627, D924 and H510 with fludioxonil (10 µg/ml) and sorbitol (1 M). Total Hog1p (lower panel, Hog1) was also detected. Hog1p appears at a site corresponding to 50 kDa.

4.7 Saturation Transfer Difference-NMR (STD-NMR) confirms the direct interaction between the CaNik1p and fludioxonil

The NMR spectrum of a saturated solution of fludioxonil in deuterated DMSO was recorded after the addition of various concentrations of the purified CaNik1p. Fig. 4.10 shows the characteristic chemical shifts of the aromatic protons of fludioxonil in the region between

7.1 and 7.6 ppm. The intensities of the fludioxonil peaks decreased proportionally according to the increase in the concentration of the purified CaNik1p in comparison with the spectrum of the sample containing fludioxonil together with the flow-through from CaNik1p ultrafiltration. This indicated the direct interaction between the compound and the protein.

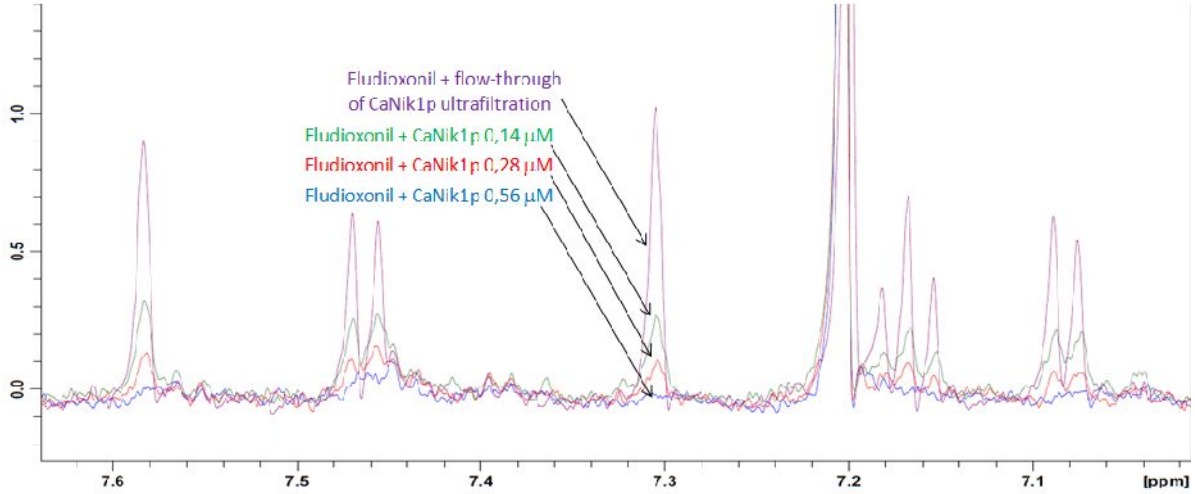


Fig. 4.10: STD-NMR spectrum showing the interaction of fludioxonil with various concentrations of purified CaNik1p.

4.8 *In vitro* kinase assay of purified CaNik1p and its mutated variants

4.8.1 Kinase-Glo Plus kit

The kinase activity of the purified CaNik1p and its mutated variants was determined by using the Kinase-Glo Plus kit as shown in Fig. 4.11. The luminescence of the samples that contained no proteins but flow-through buffer was set as 100 %, as no ATP was consumed in these samples. The kinase activity of the various purified proteins was indicated by a decrease of the ATP concentration, which resulted in decreased luminescence. From Fig. 4.11, it can be observed that all proteins possessed kinase activity. In comparison with CaNik1p, the kinase activity of CaNik1p(D924N) was decreased, whereas that of CaNik1pΔHAMP was increased by more than two-fold. Surprisingly, the point mutation H510Q in the HisKA domain slightly decreased the kinase activity of CaNik1p, whereas the point mutation N627D in the HATPase_c domain increased the kinase activity to a similar level as was observed for CaNik1pΔHAMP.

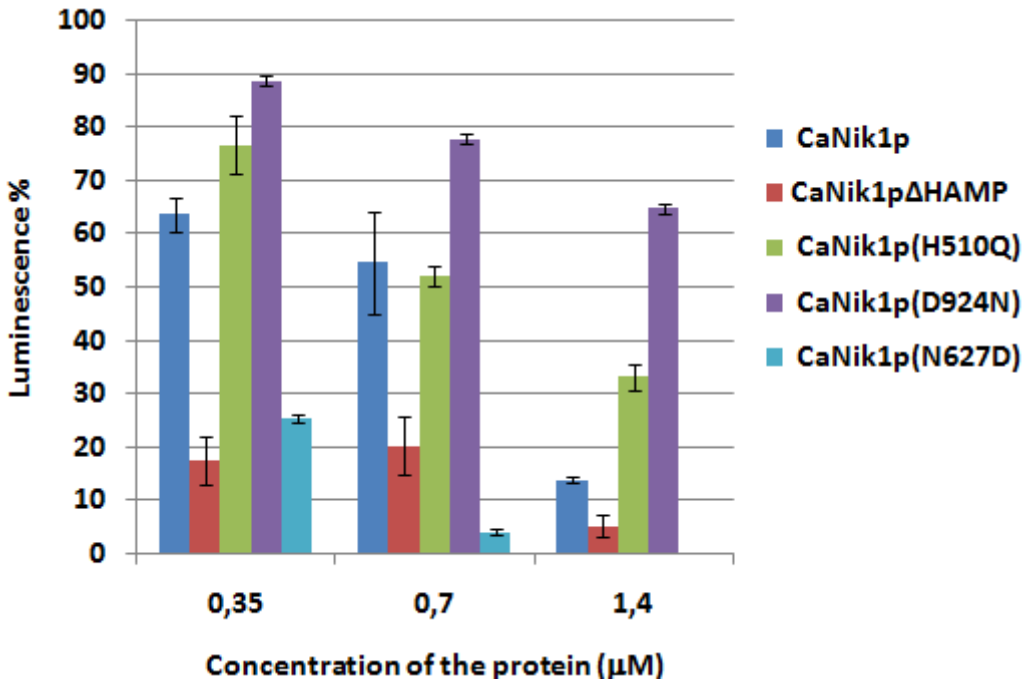


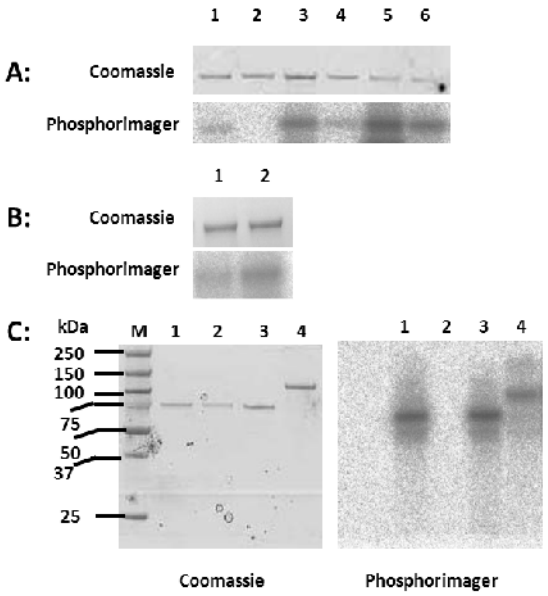
Fig. 4.11: Assay of the kinase activity of the purified CaNik1p and its mutated variants using Kinase-Glo Plus kit.

4.8.2 Detection of the autophosphorylation of the protein via radiolabeled [γ-^{32}P] ATP

As the Kinase-Glo plus assay is based on the detection of the consumption of the substrate, but not of the formation of a product, we performed kinase assays with the purified CaNik1p and its mutated variants with radiolabeled [γ-^{32}P] ATP. After scanning of the gel-exposed phosphor screens with the phosphorimage analyzer, the autophosphorylation was detected via the appearance of radioactive bands indicating the incorporation of the radiolabeled phosphate in the protein. Heat denatured protein was used as negative control. As shown in Fig. 4.12A, the autophosphorylation of the CaNik1p (lane 1) could be detected in comparison with the heat denatured CaNik1p, which showed no radioactive bands (lane 2). All point-mutated variants of CaNik1p ((H510Q), (N627D), and (D924N)) still showed autophosphorylation. Unexpectedly, even a double mutation in the conserved phosphorylatable residues, His510 and Asp924, did not inhibit the autophosphorylation of the protein (CaNik1p (H510Q, D924N)). Also the protein which was mutated in the G1 box of the HATPase_c domain (CaNik1p(G663A, G665A)) was still able to undergo autophosphorylation (Fig. 4.12B).

Deletion of HAMP domains did not inhibit the autophosphorylation of the protein, even after an additional point mutation in H510 (Fig. 4.12C). Thus, the autophosphorylation of the

purified proteins did not correlate with the antifungal susceptibility of the respective *S. cerevisiae* transformants.



Lane	Sample	Expected MW (kDa)
A:		
1	CaNik1p	121
2	CaNik1p (Heat denatured)	
3	CaNik1p(H510Q)	
4	CaNik1p(D924N)	
5	CaNik1p(N627D)	
6	CaNik1p(H510Q, D924N)	
B:		
1	CaNik1p(G663A, G665A)	121
2	CaNik1p(N627D)	
C:		
M	Marker	
1	CaNik1pΔHAMP	75
2	CaNik1pΔHAMP (Heat denatured)	
3	CaNik1pΔHAMP (H510Q)	
4	CaNik1p	121

Fig. 4.12: Detection of the autophosphorylation of the purified CaNik1p and its mutated variants using radiolabeled [γ-^{32}P] ATP.

4.8.3 Effect of fludioxonil on the *in vitro* kinase activity of the purified CaNik1p

4.8.3.1 Kinase-Glo Plus kit

The effect of fludioxonil on the kinase activity of CaNik1p was investigated by using the Kinase-Glo Plus kit. The luminescence of the samples that contained no proteins but flow-through buffer and DMSO (the solvent of fludioxonil) was set as 100 % luminescence, as no ATP was consumed in such samples. Fludioxonil was incubated with CaNik1p for 30 min at 30 °C before starting the kinase reaction by addition of ATP and further incubation at 30 °C for 2:30 h. In comparison with the luminescence obtained from the kinase reaction containing CaNik1p and DMSO, the luminescence of the kinase reaction was not affected after incubation of the protein with fludioxonil at a final concentration of 10 µg/ml (40 µM) (Fig. 4.13). This indicates that fludioxonil does not interfere with the *in vitro* kinase activity of CaNik1p.

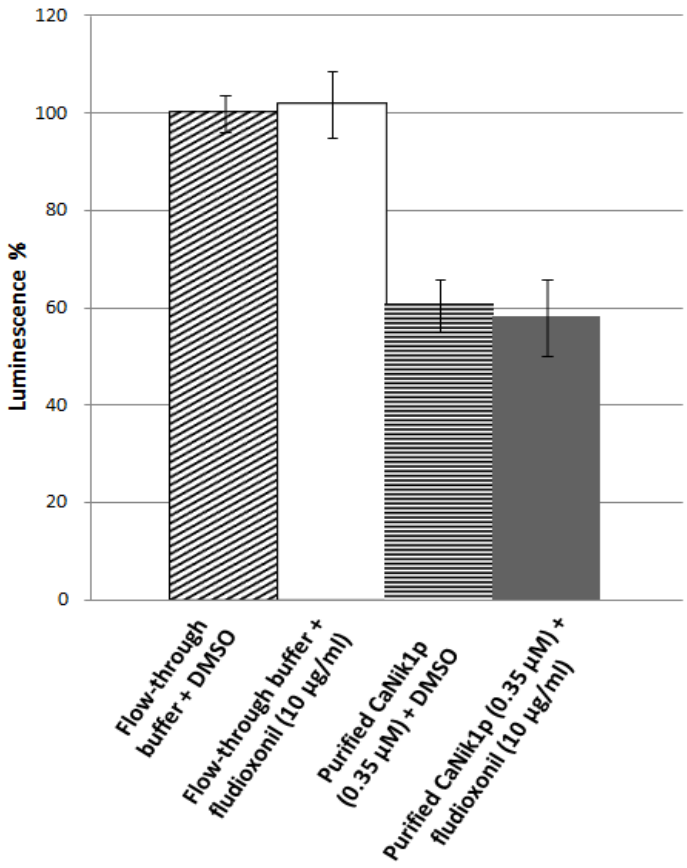
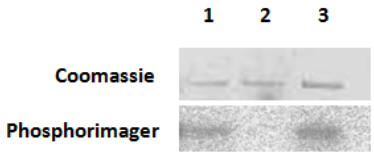


Fig. 4.13: Determination of the effect of fludioxonil on the kinase activity of purified CaNik1p using Kinase-Glo Plus kit.

4.8.3.2 Radiolabeled [γ-^{32}P] ATP

The effect of fludioxonil on the autophosphorylation of CaNik1p was also investigated via the detection of phosphorylated protein after incubation with radiolabeled [γ-^{32}P] ATP. Fludioxonil was incubated with CaNik1p at 30 °C for 30 min before starting the kinase reaction by addition of both cold and hot ATP and further incubation at 30 °C for 1 h. As demonstrated in Fig. 4.14, the intensity of the radioactive bands in the sample containing CaNik1p and DMSO (the intensity indicates the incorporation of the radiolabeled phosphate in the protein via the kinase reaction) was not affected by the incubation of the protein with fludioxonil at a final concentration of 10 µg/ml. This shows that fludioxonil does not interfere with the autophosphorylation of CaNik1p.



Lane	Sample	Expected MW (kDa)
1	CaNik1p + DMSO	121
2	CaNik1p (Heat denatured)	
3	CaNik1p + Fludioxonil (10µg/ml)	

Fig. 4.14: Determination of the effect of fludioxonil on the autophosphorylation of the purified CaNik1p using radiolabeled [γ-^{32}P] ATP.

4.9 LC-MS/MS peptide analysis showed that the Ser1071 is autophosphorylated

The possibility of the phosphorylation of residues other than H510 and Asp924 in CaNik1p was investigated by LC-MS/MS peptide analysis. The purified CaNik1p (1.5 µg, 1.25 µM) was incubated in 10 µl kinase buffer A at 30 °C, either with or without 20 µM ATP. After 2:30 h, the kinase reaction was terminated by boiling at 95 °C with 5x Laemmli buffer. Both protein samples were then separated by SDS-PAGE, and bands of the protein at the expected MW were excised and sent for LC-MS/MS peptide analysis as mentioned in 3.2.10.

The analysis of the possible phosphorylation of the different residues in the various peptides was performed by Dr. Josef Wissing and Dr. Manfred Nimtz (Cellular Proteome Research unit, HZI). Only Ser1071 (Fig. 4.15) was identified as being phosphorylated with a probability of more than 99.1 %. However this phosphorylation was not dependent on the presence of ATP. It has to be taken into account that sample pretreatment includes treatment with acidic solution. Under these conditions, phosphorylated histidine or aspartic acid is not stable and thus the phosphorylated state of these residues cannot be evaluated by the applied method.

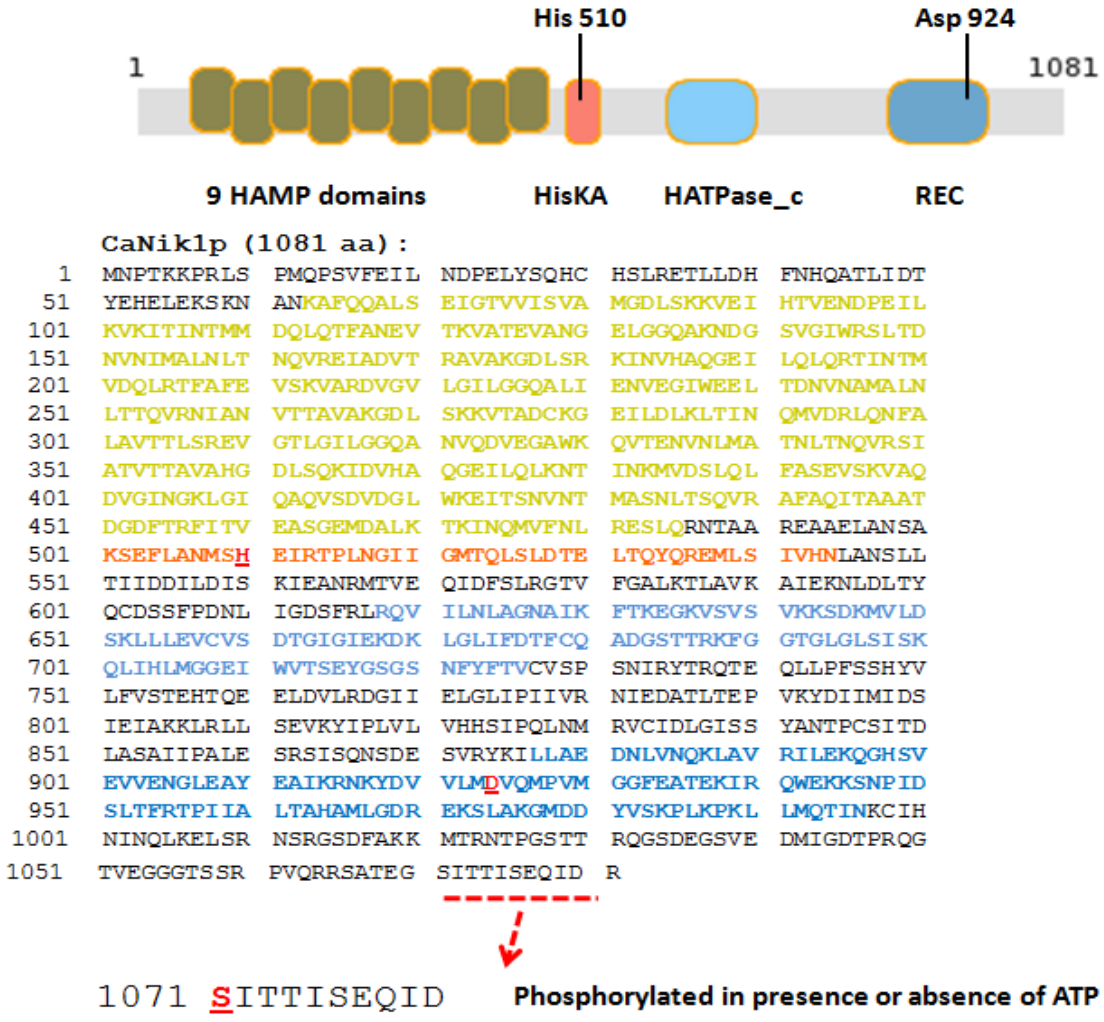


Fig. 4.15: Schematic diagram of CaNik1p and its corresponding sequence indicating the conserved phosphorylatable residues and the newly identified Ser1071.

4.10 Deletion of HAMP domains from CaNik1p

4.10.1 Deletion of HAMP domains in the transformants led to growth inhibition that could be reversed by point mutation of the His510

Transforming *S. cerevisiae* with *CaNIK1* variants in which all the HAMP domains were deleted resulted in the ΔHa and ΔHupa strains which were able to grow on SD-ura agar plates, where expression of the mutated variants of CaNik1p proteins is not induced (Fig. 4.16). Surprisingly no growth was observed on SG-ura plates on which galactose induced the expression of the CaNik1pΔHAMPup and CaNik1pΔHAMP (Fig. 4.16), whereas deletion of two pairs of HAMP domains did not affect the growth of the transformed strain ΔH3H4 (47) (Fig. 4.16B). Additional inactivation of the HisKA domain by the H510Q point mutation

restored normal growth of the resultant transformed strains ΔHaH510 and ΔHupaH510 (Fig. 4.16).

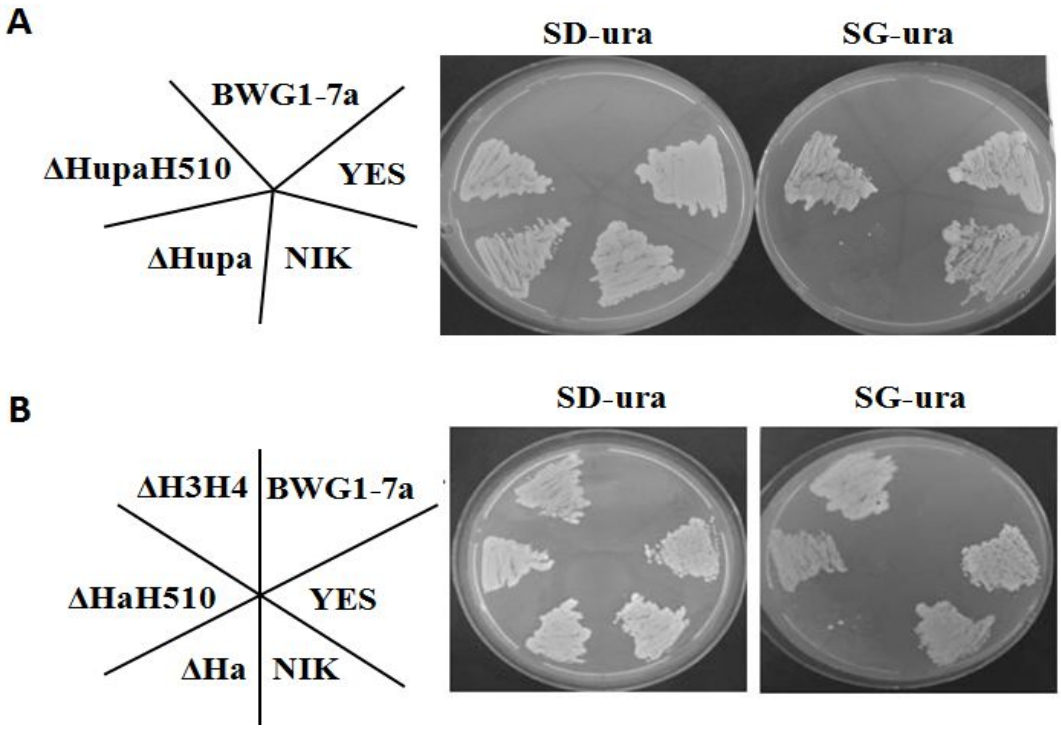


Fig. 4.16: Growth inhibitory effect resulting from the expression of CaNik1pΔHAMPup and CaNik1pΔHAMP in the transformed *S. cerevisiae* could be reversed by the additional point mutation H510Q, as shown in A and B, respectively. Strains BWG1-7a, YES, NIK, ΔHupa, ΔHupaH510, ΔHa, ΔHaH510, and ΔH3H4 were streaked on SD-ura and SG-ura agar plates and incubated at 30 °C for 4 days. SG-ura induces the expression of the transgenes via the galactose promoter. Strain BWG1-7a is the reference strain, which is auxotrophic for uracil.

4.10.2 The growth inhibitory effect could be reversed in the deletion mutants of the response regulator Ssk1p and components of the Hog1 MAPK module

Transformation of *S. cerevisiae* strains, in which individual genes are deleted that either encode the RR Ssk1p (strain Δssk1), or one of the components of the Hog1 MAPK module, such as the MAP2K Pbs2p (strain Δpbs2) and the MAPK Hog1p (strain Δhog) with the plasmids pYES2-*CaNIK1*ΔHAMPup and pYES2-*CaNIK1*ΔHAMP restored normal growth on SG-ura plates (Fig. 4.17A and Fig. 4.17B). The reference strain for such mutants was BY4741. Therefore, as a control, this strain was also transformed with the plasmids pYES2-*CaNIK1*ΔHAMPup (strain ΔHupb) and pYES2-*CaNIK1*ΔHAMP (strain ΔHb). Following transformation, the strain showed the same phenotype (Fig. 4.17) as that observed with the

reference strain BWG1-7a in Fig. 4.16. In addition, simultaneous H510Q point mutation restored normal growth of the resultant transformed strains ΔHbH510 and ΔHupbH510 (Fig. 4.17).

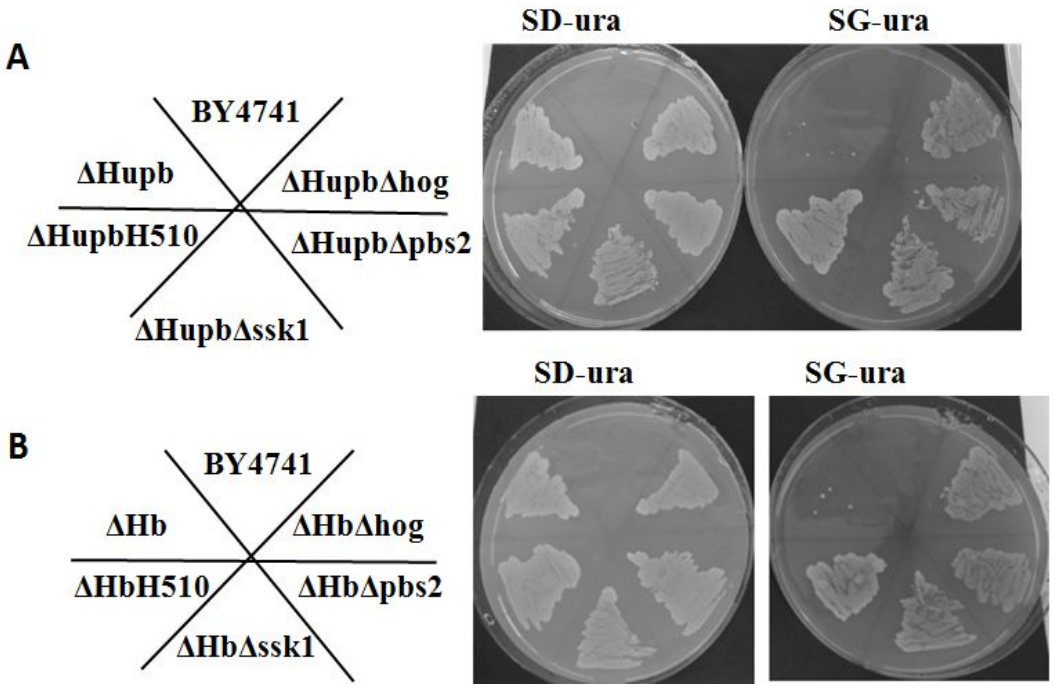


Fig. 4.17: Growth inhibitory effect resulting from the deletion of HAMP domains could be reversed by transforming *S. cerevisiae* containing single gene deletion mutants in one of the components of the Hog1 MAPK module or in the RR *SSK1*. A and B present transformants with the plasmids pYES2-*CaNIK1*ΔHAMPup and pYES2-*CaNIK1*ΔHAMP, respectively. Strains BY4741, ΔHupbΔhog, ΔHupbΔpbs2, ΔHupbΔssk1, ΔHupbH510, ΔHupb, ΔHbΔhog, ΔHbΔpbs2, ΔHbΔssk1, ΔHbH510, and ΔHb were streaked onto SD-ura and SG-ura agar plates and incubated at 30 °C for 4 days. BY4741 is the reference strain, which is auxotrophic for uracil.

4.10.3 Expression of CaNiK1pΔHAMP leads to constitutive phosphorylation of the Hog1p

To analyze further the involvement of Hog1p activity in the growth inhibitory effect associated with the induction of expression of CaNik1pΔHAMP, the phosphorylation state of Hog1p was investigated. Because of the growth inhibitory effect of CaNik1pΔHAMP expression, the transformant ΔHa was first cultivated on the glucose–containing medium SD-ura, which does not induce CaNik1pΔHAMP expression, in order to produce sufficient biomass for protein analysis. Subsequently, the expression of CaNik1pΔHAMP was induced

by incubating the cells in the galactose–containing medium SG-ura. Gene expression and protein synthesis were allowed to occur for 180 min before fludioxonil was added. Expression of CaNik1pΔHAMP was established by Western blot (Fig. 4.18). Phosphorylation of Hog1p was examined after another 15 min and 30 min (in total 195 min and 210 min respectively) (Fig. 4.19). After fludioxonil treatment, phosphorylation of Hog1p was observed in the transformant NIK1 carrying the full-length protein and in the transformant ΔHa, whereas no phosphorylation was detected in the strains with the empty plasmid (YES) or with the additional H510Q mutation (ΔHaH510). Hog1p was phosphorylated in the transformant ΔHa, even without the presence of fludioxonil, whereas such constitutive phosphorylation was not observed in the transformants NIK and ΔHaH510 (Fig. 4.19).

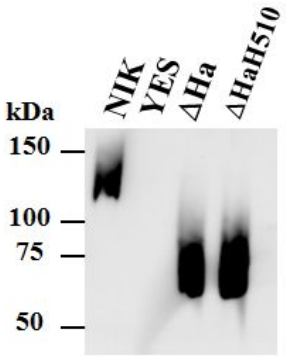


Fig. 4.18: Confirming the expression of CaNik1pΔHAMP from strain ΔHa after cultivation in SG-ura by Western blot. The expressed CaNik1p, CaNik1pΔHAMP, and CaNik1pΔHAMP(H510Q) in the isolated protein extracts from the strains NIK, ΔHa, and ΔHaH510, respectively, were detected by Western blot by using an anti-Flag antibody. The strains were cultivated in SG-ura for 180 min. CaNik1p, CaNik1pΔHAMP, and CaNik1pΔHAMP(H510Q) appear at sites corresponding to 121, 75, and 75 kDa, respectively.

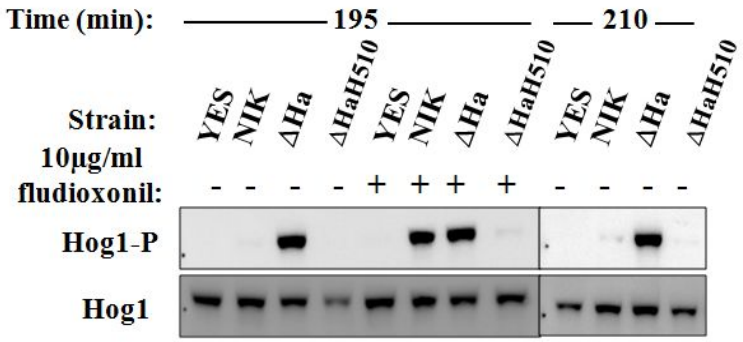


Fig. 4.19: Detection of phosphorylation of Hog1p by Western blot in the strain ΔHa. The phosphorylation of Hog1p (upper panel, Hog1-P) was detected in strains YES, NIK, ΔHa, and ΔHaH510 after cultivation in SG-ura for 195 and 210 min. Fludioxonil was added after 180 min at a final concentration of 10 µg/ml. Total Hog1p (lower panel) was also detected. Hog1p appears at a site corresponding to 50 kDa.

4.11 Deletion and reintegration of the *CaNIK1* gene in *C. albicans* Sc5314

4.11.1 Construction of the *CaNIK1* deletion cassette

The *SAT1* flipper cassette (Fig. 4.20A) was used in the construction of the *CaNIK1* deletion cassette (Fig. 4.20C). Basically, the upstream (-448 to -1) and downstream (+3247 to +3750) sequences flanking *CaNIK1* (+1 to +3246) (Fig. 4.20B) were amplified by PCR from the genomic DNA of Sc5314 and ligated to the *SAT1* flipper cassette via the In-Fusion cloning enzyme.

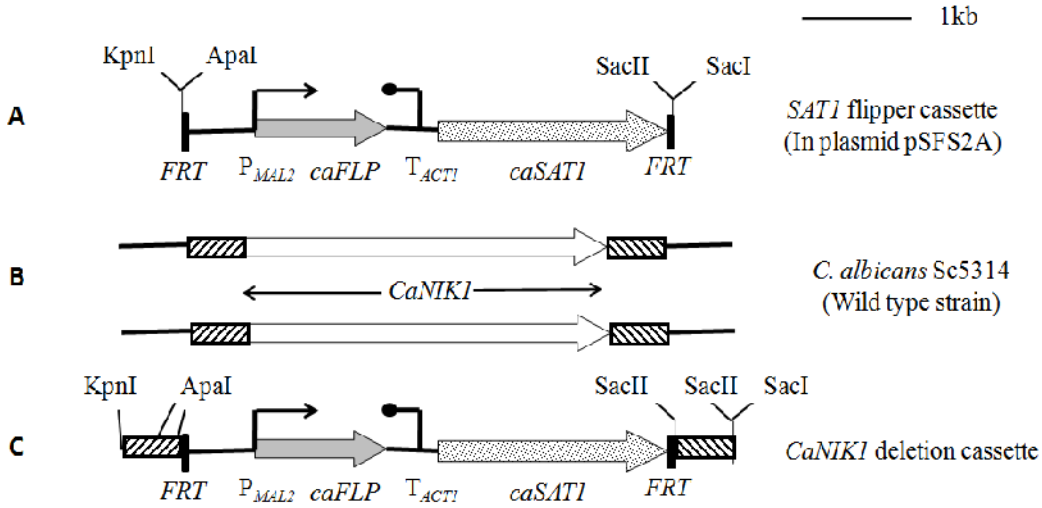


Fig. 4.20: Schematic representation of the design of the *CaNIK1* deletion cassette.

The plasmid pSFS2A (Fig. 4.21) was digested with KpnI, ApaI and SacII to yield the *SAT1* flipper cassette and the remaining backbone of the pSFS2A plasmid. The *CaNIK1*-upstream sequence was amplified with the primers F1NIK and R1bNIK, which share 15 bp of homology with the ends of the KpnI- and ApaI-restricted pSFS2A, respectively. The *CaNIK1*-downstream sequence was amplified with the primers F2NIK and R2NIK, which included 15 bp of overlap with both termini of the SacII-digested pSFS2A.

The PCR fragments representing the *CaNIK1*-upstream and -downstream sequences were purified from the agarose gel (Fig. 4.22) and ligated with the two fragments resulting from digestion of pSFS2A with KpnI, ApaI and SacII via the In-Fusion enzyme to yield the plasmid pNIK (Fig. 4.21).

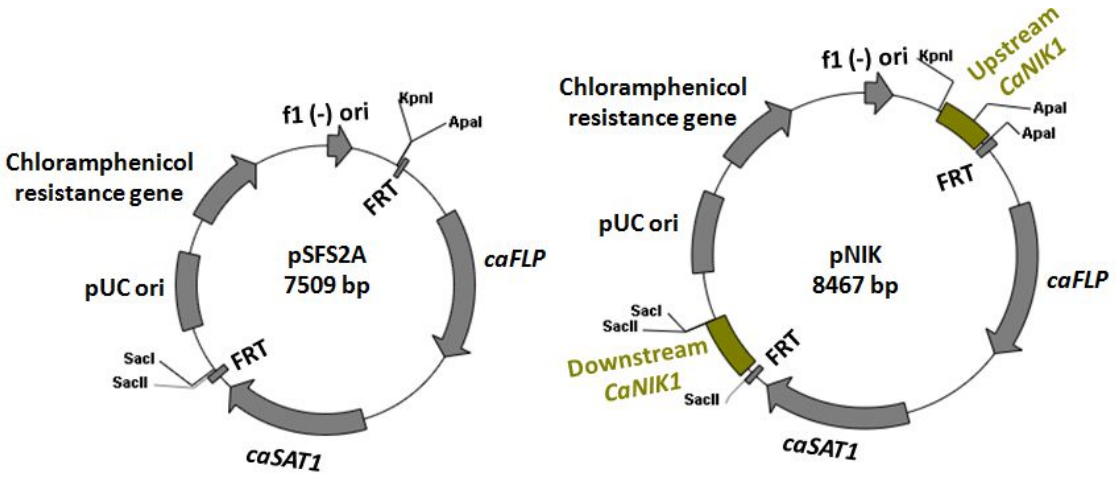
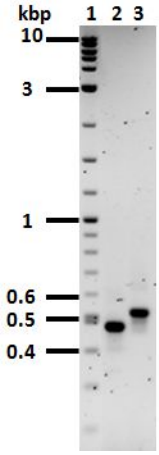


Fig. 4.21: Maps of the pSFS2A and pNIK plasmids.

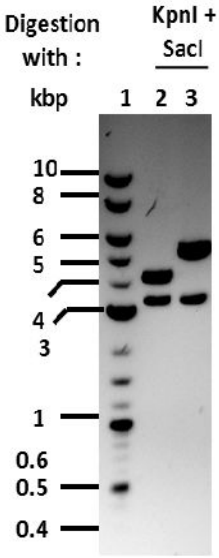


Lane	Sample	Expected size (bp)
1	Marker	-
2	*CaNIK1*-upstream sequence	480
3	*CaNIK1*-downstream sequence	536

Fig. 4.22: PCR-amplified *CaNIK1*-upstream and -downstream flanking sequences that were used for construction of the *CaNIK1* deletion cassette.

4.11.2 Confirmation of the constructed plasmid pNIK by restriction digestion

The constructed plasmid pNIK (Fig. 4.21) which comprises the *CaNIK1* deletion cassette was confirmed with regard to its integrity and correct insertion via digestion with KpnI and SacI (Fig. 4.23). Subsequently, it was further confirmed by sequencing.



Lane	Sample	Expected size (bp)
1	Marker	-
2	pSFS2A	4211 and 3298
3	pNIK	5169 and 3298

Fig. 4.23: Restriction digestion of the plasmid pNIK in comparison with pSFS2A.

4.11.3 Integration of the *CaNIK1* deletion cassette into the genome of *C. albicans* Sc5314

The *SAT1* flipper cassette (74) was used as basis for the construction of the *CaNIK1* deletion cassette (as indicated in 4.11.1). The principle of the cassette is shown in Fig. 4.24. The *SAT1*-flipping strategy allows gene deletion in prototrophic *C. albicans* wild-type strains with the help of a recyclable dominant selection marker. The *SAT1* flipper cassette consists of the *caSAT1* marker, which confers resistance to the antibiotic NST, and the *caFLP* gene, which encodes the site-specific recombinase FLP. The addition of flanking sequences of the target gene allows specific genomic insertion of the *SAT1* flipper cassette by homologous recombination (Fig. 4.24A) and selection of NST-resistant transformants, which are able to grow in the presence of high concentration of NST (200 µg/ml). Expression of the FLP recombinase results in the subsequent excision of the cassette, which is bordered by direct repeats of the FLP recognition sequence *FRT*, from the genome of the wild-type strain (Fig. 4.24B). Therefore, the cells that undergo flip excision will grow slowly (NST-sensitive), as small colonies, on YPD agar plates containing a low concentration of NST (≤ 20 µg/ml) in comparison with the cells that do not undergo flip excision and will grow as large colonies

(NST resistant). The heterozygous mutant (NST sensitive) can then be used for a second round of gene deletion (Fig. 4.24C). The homozygous mutant (Fig. 4.24D) obtained after two rounds of insertions and recycling of the *SAT1* flipper cassette differs from the wild-type parental strain only by the absence of the target gene and can be used for the inactivation of additional genes and the generation of complemented strains by using the same strategy (74).

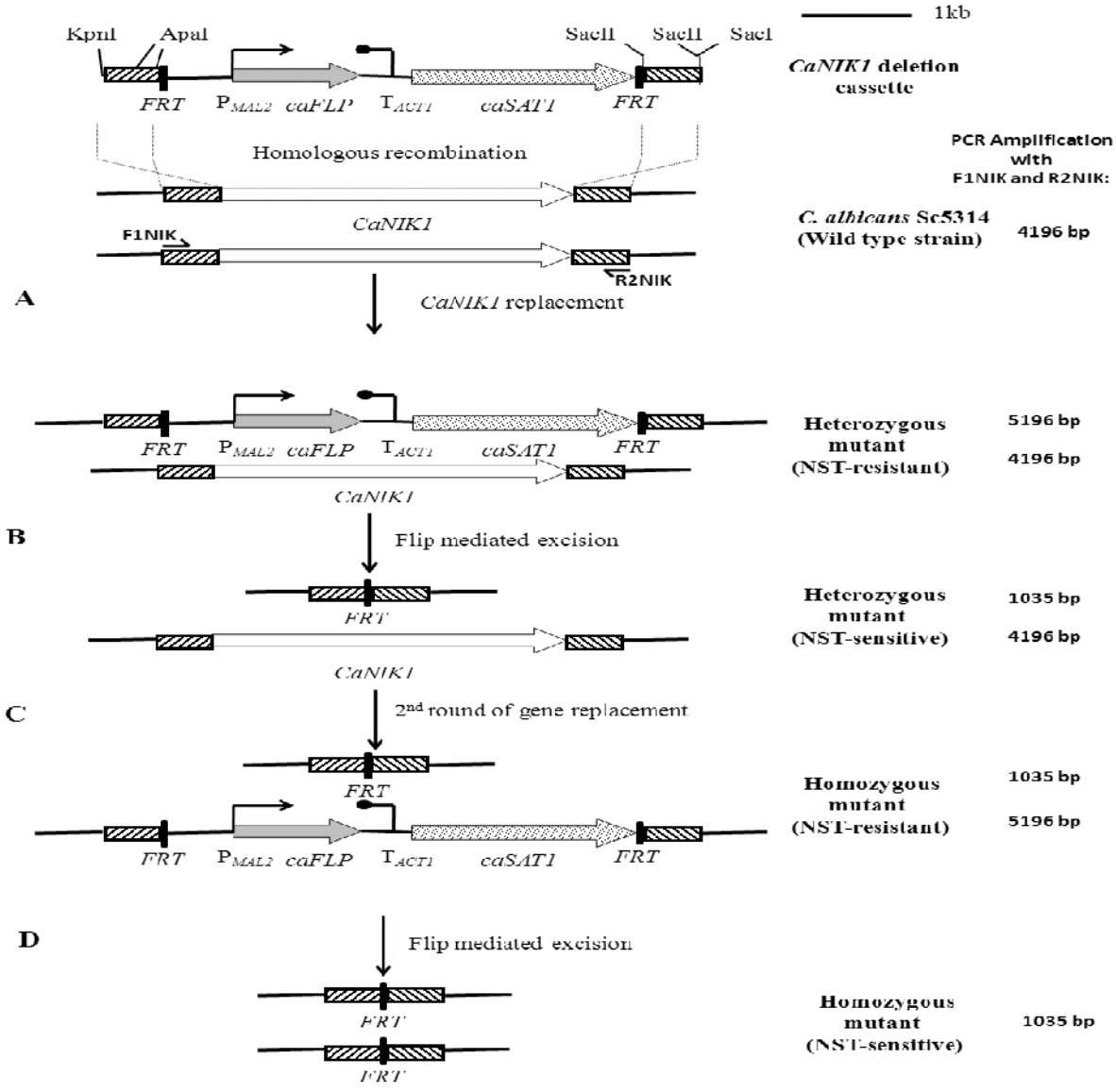
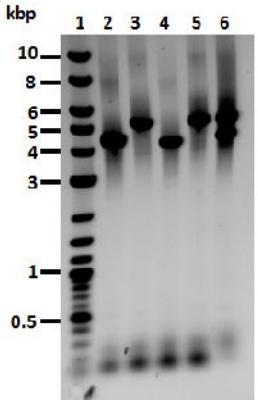


Fig. 4.24: The principle of deletion of the *CaNIK1* gene in the wild strain Sc5314 using the *SAT1* flipper method. The expected sizes after PCR amplification of the genomic DNA of the wild strain Sc5314 and the resulting mutants using the primers F1NIK and R2NIK are given on the right side.

The wild strain Sc5314 was transformed by electroporation with the *CaNIK1* deletion cassette that was obtained by digestion of the plasmid pNIK with KpnI and SacI. The electroporated cells were spread on YPD agar plates containing 200 µg/ml NST as a selection marker. After 2 days of incubation, some of the resulting colonies were analyzed by colony PCR with the primers F1NIK and R2NIK to check whether the *CaNIK1* deletion cassette had been integrated successfully into the *CaNIK1* gene (Fig. 4.25). PCR showed that the colony NIKd underwent integration in a single allele, whereas the colonies NIKa and NIKc appeared to undergo integration in both alleles of the *CaNIK1* gene in a single step. On the other hand, the colony NIKb did not undergo homologous recombination.



Lane	Sample	Expected size (bp)
1	Marker	-
2	Genomic DNA of Sc5314	4196
3-6	-Colony PCR for some colonies that grew on NST-YPD agar plates (200 µg/ml). -Lanes 3, 4, 5 and 6 are colonies NIKa, NIKb, NIKc, and NIKd, respectively.	-5196 and 4196 (Integration in single allele of *CaNIK1*). -5196 (Integration in both alleles of *CaNIK1*). - 4196 (No integration occurs).

Fig. 4.25: Colony PCR of electroporated Sc5314 with the *CaNIK1* deletion cassette after growing on NST-YPD agar plates (200 µg/ml).

4.11.4 Excision of the *SAT1* flipper cassette

In the *SAT1* flipper cassette, the site-specific recombinase FLP is under the control of the inducible promoter *MAL2*. Therefore, for the recycling of the *SAT1* flipper cassette (Fig. 4.24B and D), the transformants should be cultivated in YPM medium that contains maltose instead of glucose. However, the *MAL2* promoter is leaky, and the FLP recombinase is also expressed in YPD medium (74). Therefore, recycling of the *SAT1* flipper cassette can also be achieved by growing the transformants overnight in YPD medium without selective pressure (74).

Colonies NIKa and NIKd were selected for further genetic manipulation. For recycling of the *SAT1* flipper cassette, the transformant NIKd was cultivated in YPD medium overnight and the cells were streaked on YPD agar plates containing various concentrations of NST (20, 10, 5 µg/ml) before incubation at 30 °C for 2 days. Small colonies (NST sensitive), which should represent the cells that had undergone flip excision, could be observed only on the NST plates at 10 and 5 µg/ml, whereas that of 20 µg/ml showed only large colonies. Several

small colonies (NIKd1-NIKd10) were selected from the NST plates (10 µg/ml) (Fig. 4.26) and analyzed by colony PCR for the induction of the FLP recombinase by using the primer sets (F1NIK and R2NIK) and (F1SAT and R1SAT). As shown in Fig. 4.27A, only one colony (NIKd1) underwent flip excision. However, the colony still contained the *SAT1* flipper cassette. Moreover, and confirming these result, the colony NIKd1 was able to grow either on solid or in liquid YPD medium containing NST at a concentration of 200 µg/ml.

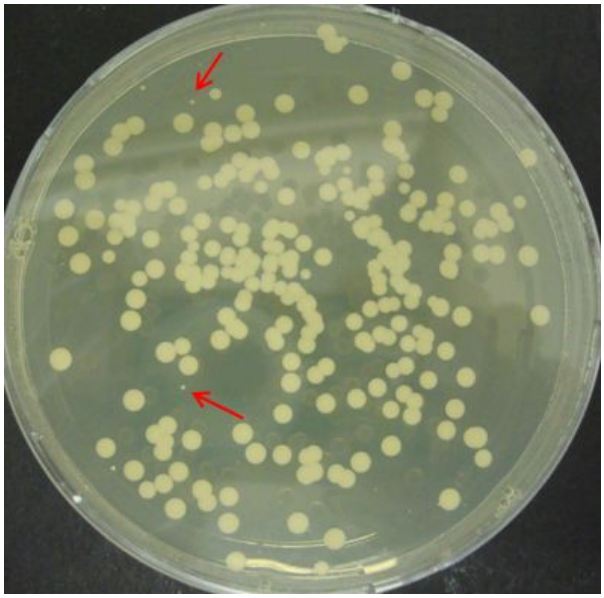


Fig. 4.26: Selection and screening for NST-sensitive *C. albicans* transformants in which the *SAT1* flipper cassette was excised by FLP-mediated recombination. The plate shows large colonies of NST-resistant transformant and small colonies of NST-sensitive derivatives (indicated by arrows) which, after the induction of FLP expression, were grown for 2 days at 30 °C on a YPD plate containing 10 µg/ml NST.

For the recycling of the *SAT1* flipper cassette in the transformant NIKa, the transformant was cultivated in YPD medium in 5 repetitive overnight cultures (each overnight culture was prepared from the previous one), and finally the cells were streaked on YPD agar plates containing various concentrations of NST (5, 10, 20 µg/ml) before incubation at 30°C for 2 days. These consecutive overnight cultures were essential to ensure that the flip excision occurred in both alleles of the transformant. No colonies were observed on the NST plates at 10 and 20 µg/ml, whereas several small colonies could be observed on the NST plate at 5 µg/ml. A single colony (NIKhom2) was selected for further analysis. The cells of NIKhom2 were unable to grow in the presence of a high concentration of NST (200 µg/ ml). In addition, PCR analysis (Fig. 4.27B) revealed the successful recycling of the *SAT1* flipper in both copies

of the integrated *CaNIK1* deletion cassette. Therefore, the NIKhom2 mutant could be used for further complementation analysis of *CaNIK1* and its mutated variants.

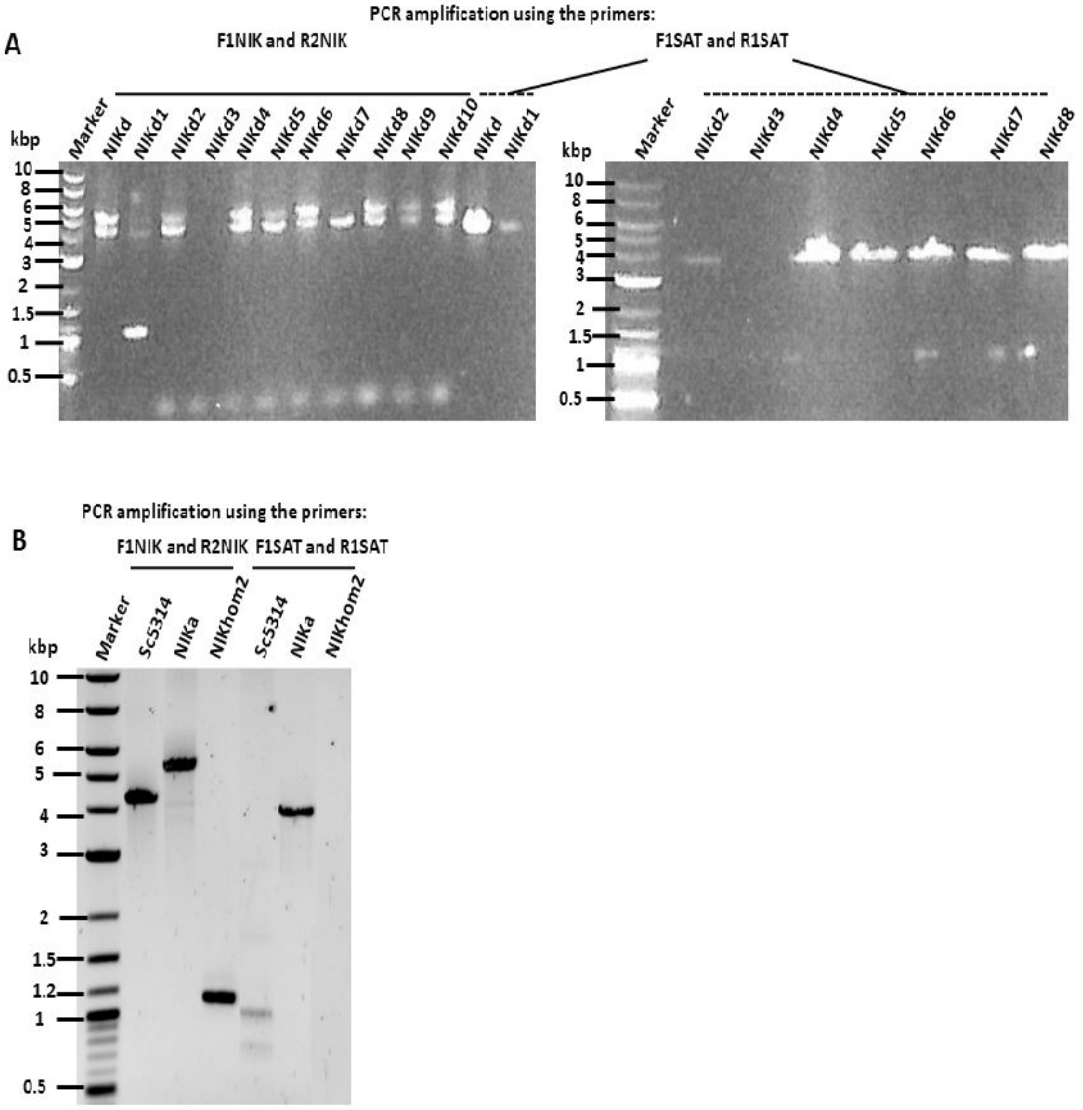


Fig. 4.27: PCR screening of NST-sensitive derivatives that underwent FLIP excision after growing colonies NIKd (A) and NIKa (B) in YPD medium without selective pressure. The expected sizes when using the set of primers F1NIK and R2NIK are illustrated in Fig. 4.24, whereas for F1SAT and R1SAT it is 3768 bp.

4.11.5 Reintegration of *CaNIK1* and some of its mutated variants

To study the effect of the (H510Q) mutation and deletion of HAMP domains of the *CaNIK1* gene in *C. albicans*, we planned to construct *CaNIK1* reintegration cassettes. These cassettes should have contained the *CaNIK1*-tagged gene and its mutated variants that could

be PCR amplified from the different plasmids that harbored different mutations of the *CaNIK1* and were used in the transformation of *S. cerevisiae.*

The *SAT1* flipper cassette was used for the construction of the reintegration cassettes of *CaNIK1* and its mutated variants. These could be used for complementation of the *CaNIK1* (Fig. 4.28) or incorporation of its mutated variants into the homozygous mutant of *CaNIK1* (NIKhom2).

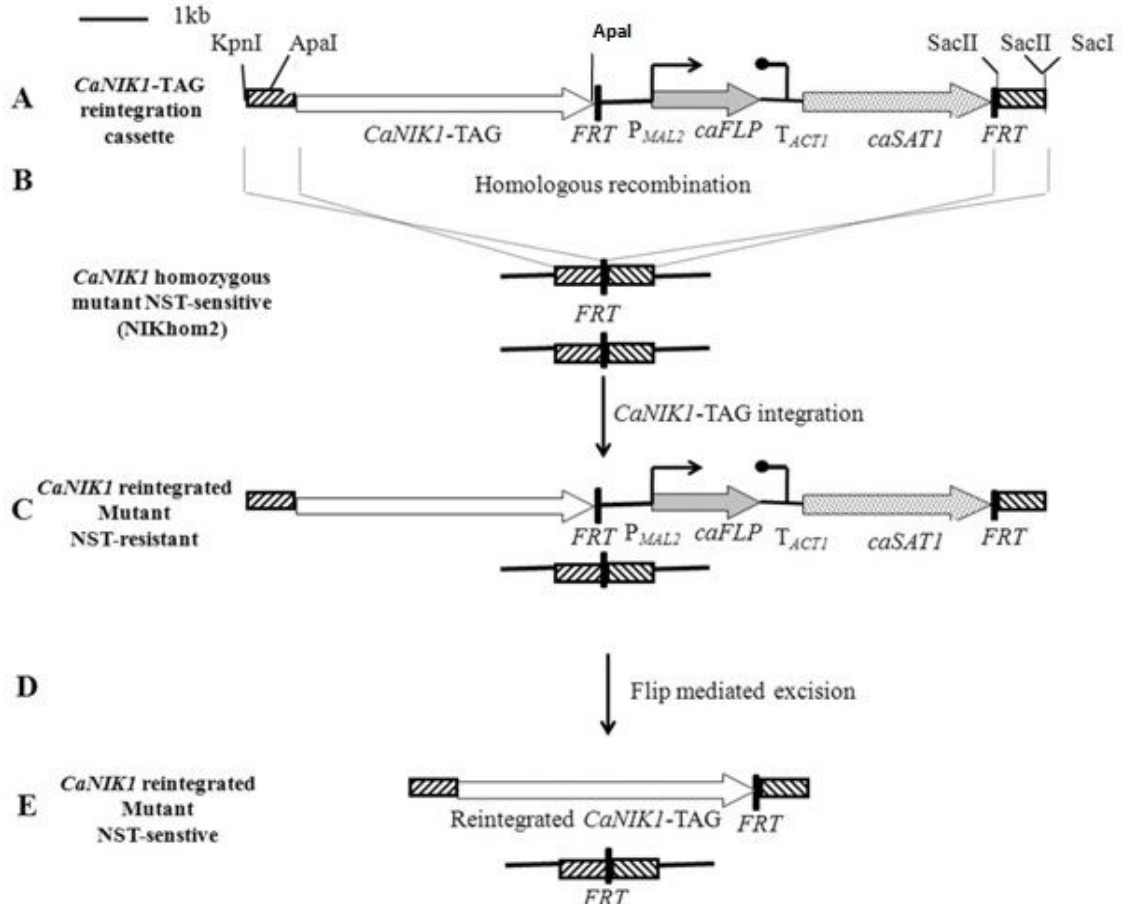
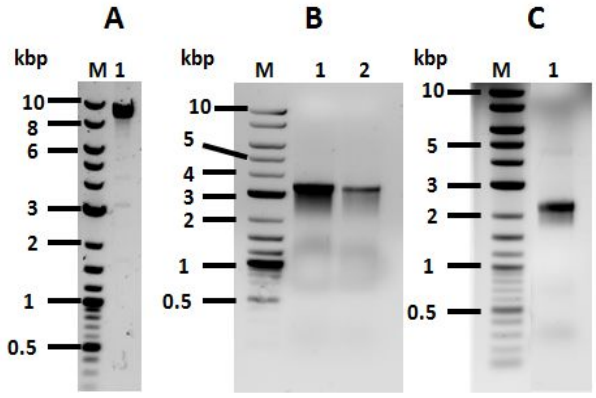


Fig. 4.28: Schematic representation of the planned reintegration of *CaNIK1*-TAG into the genome of the homozygous deletion mutant of *CaNIK1*.

To construct the *CaNIK1*-TAG reintegration cassette, the *SAT1* cassette should be flanked from the upstream side by both the *CaNIK1* gene in addition to its upstream sequence and from the downstream side by the downstream sequence of the *CaNIK1* gene as shown in Fig. 4.28A.

The whole pNIK was amplified by PCR by using the primers F4NIK and R3NIK (Fig. 4.29A, Fig. 4.30A). R3NIK had an additional 15 bp of homology to the first 15 bp of the *CaNIK1* gene. *CaNIK1*-TAG, *CaNIK1*(H510Q), and *CaNIK1*ΔHAMP were amplified from the plasmids pYES2-*CaNIK1*-TAG, pYES2-*CaNIK1*(H510Q), and pYES2-*CaNIK1*ΔHAMP,

respectively, by using the primers F3NIK and R5NIK (Fig. 4.29B and C). The primer R5NIK shared 15 bp of homology with the F4NIK primer.



Lane	Sample	Expected size (bp)
M	Marker	-
A:		
1	pNIK	8467
B:		
1	pYES2-*CaNIK1*-TAG	3332
2	pYES2-*CaNIK1*(H510Q)	
C:		
1	pYES2-*CaNIK1*ΔHAMP	2063

Fig. 4.29: PCR amplification of various fragments required to construct the integration cassettes of the *CaNIK1*-TAG and its mutated variants. The primers used for the PCR were F4NIK and R3NIK for sample in gel A, whereas F3NIK and R5NIK were used for samples in gel B and C.

To construct the plasmid that would contain the reintegration cassettes (Fig. 4.30B), the In-Fusion enzyme was used to ligate the PCR amplified pNIK with *CaNIK1*-TAG. Before fusion of the previous fragments, digestion of the PCR amplified pNIK with DpnI was performed to avoid interference in the transformation of the competent cells from the pNIK that was used as template in the PCR. After incubation of the DNA fragments with the In-Fusion enzyme, the mixture was used to transform Stellar competent *E. coli* cells and the transformed cells were streaked on LB agar plates containing chloramphenicol (170 µg/ml). Some of the resulting colonies were cultivated in LB medium containing chloramphenicol (170 µg/ml), and plasmids were isolated from the overnight cultures and digested with KpnI and SacI to test for successful ligation of the fragments with In-Fusion enzyme. Analysis of the plasmids by restriction enzyme digestion (Fig. 4.31) showed that the ligation was unsuccessful, in addition the resulting colonies, represented cells that were transformed with DpnI-undigested pNIK.

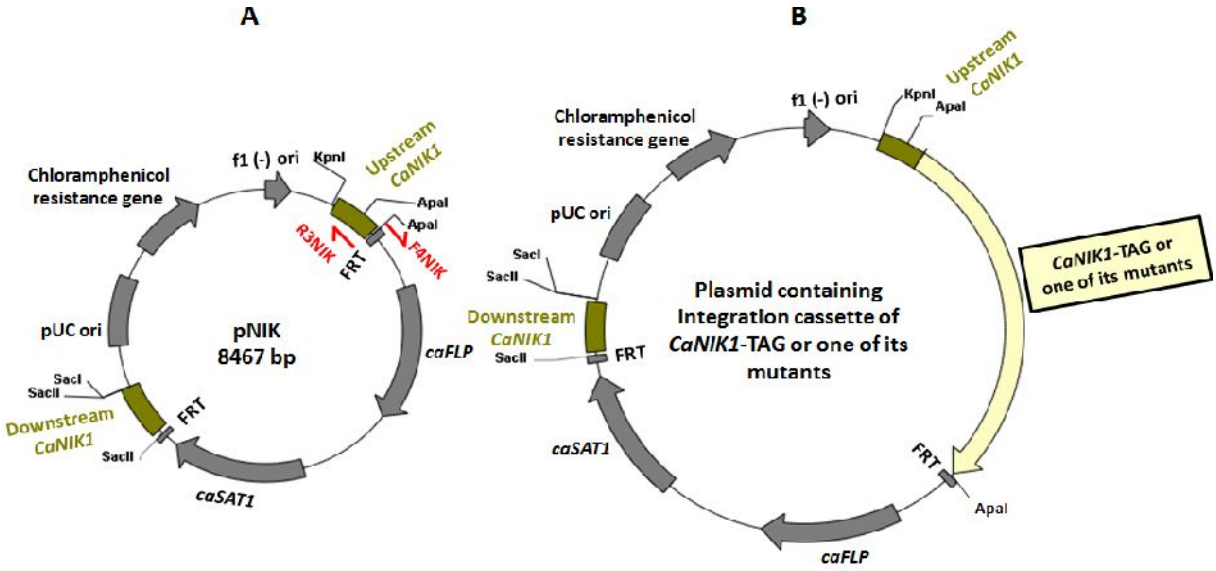
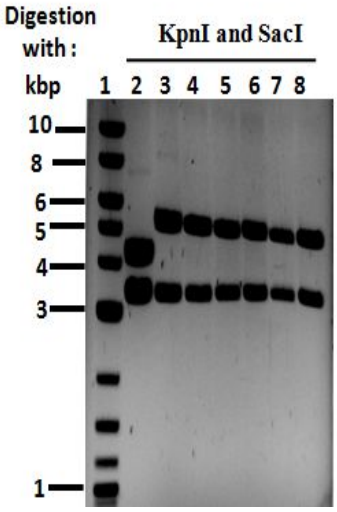


Fig. 4.30: Map of pNIK showing the binding sites of the primers F4NIK and R3NIK and map of the plasmids supposed to harbor the *CaNIK1* reintegration cassettes as indicated in A and B, respectively.



Lane	Sample	Expected size (bp)
1	Marker	-
2	pSFS2A	4211 and 3298
3	pNIK	5169 and 3298
4-8	Plasmids isolated from cultures of different colonies that grew on LB plates containing chloramphenicol (170 µg/ml)	8500 and 3298

Fig. 4.31: Restriction enzyme digestion of plasmids supposed to contain the *CaNIK1*-TAG reintegration cassettes.

5 Discussion

5.1 Relevance of constructing mutations in the heterologously expressed CaNik1p

The sensitivity of *C. albicans* to the antifungals targeting the Hog1 MAPK module depends on the genetic background of the strain. Janine Wesolowski showed that strain CAF2-1 was resistant to these fungicides, whereas strain 1386 was sensitive to the tested antifungals (90). This could be explained by the development of resistance to these antifungals (91).

Because of the diploid nature of *C. albicans*, targeted point mutations or deletions of defined protein domains still represent a technical challenge. As *S. cerevisiae* acquires susceptibility to various antifungals when transformed with a plasmid encoding *CaNIK1*, this has encouraged further use of the same transfectant as a model organism to investigate the involvement of various CaNik1p domains in fungicidal activity. To achieve this goal, point mutations were introduced in the HisKA, HATPase_c, and REC domains of CaNik1p. In addition, a deletion mutant for all HAMP domains was generated. The plasmids harboring the mutated variants of *CaNIK1* were used to transform *S. cerevisiae* followed by testing viability and determining sensitivity to fungicides and phosphorylation of the MAPK Hog1p upon fungicidal treatment.

5.2 Effect of point mutations of the heterologously expressed CaNik1p

5.2.1 Sensitivity of the transformants to the antifungals

We previously showed that expression of the group III HK from the human fungal pathogen *C. albicans, CaNIK1,* in *S. cerevisiae* resulted in susceptibility of the transformants to the fungicides fludioxonil, iprodione, and ambruticin VS3 (47). For other group III HKs, mutations in the conserved phosphate-accepting residues of the HisKA and the REC domains had previously been shown to confer fungicide resistance (46, 48). Thus, we were interested to investigate the role of the HisKA, HATPase_c, and REC domains from CaNik1p in fungicide activity, since they are conserved in all HKs. However, only class III HKs were found to be important for fungicide activity.

To prevent the primary phosphorylation of the histidine residue and the subsequent His-Asp phosphate-transfer in the HisKA and the REC domains, respectively, the point mutations H510Q and D924N were generated. The N627D mutation and the (G663A, G665A) double mutation were supposed to inactivate the ATP-binding site (HATPase_c). The obtained

results were in agreement with those obtained from other group III HKs, as the functionality of the above-mentioned domains was essential for the sensitivity of the transformed yeast for the tested fungicides, as demonstrated by the complete resistance of strains H510 and D924 in comparison with strain NIK and by the reduced or complete loss of the fungicidal sensitivity of strains N627 and G1. The single replacement of the Asn residue in the N box appears to be insufficient to completely inhibit ATP binding, contrary to the double mutation of the Gly residues in the G1 box.

5.2.2 Phosphorylation of the Hog1p after treatment of the transformants with fludioxonil

In agreement with the fungicidal susceptibility of the various constructed transformants, similar patterns of Hog1p phosphorylation were obtained after treatment of such transformants with fludioxonil. Phosphorylation was totally abolished in strains H510 and D924 and partially inhibited in strain N627. We present clear evidence that the conserved phosphorylatable residues of CaNik1p, His510, and Asp924 are essential for fungicidal activity and for the activation of the Hog1 MAPK module resulting from treatment with fungicide.

5.3 The direct interaction of fludioxonil with the CaNik1p

Heterologous functional expression of CaNik1p and other group III HKs enabled the transfer of fungicidal sensitivity to *S. cerevisiae* establishing the essential role of this class of HKs for antifungal activity (45-48). However, no clarification had been obtained as to whether group III HKs are the direct targets of the fungicides or mediators of their action, leading finally to the activation of the Hog1 MAPK module and the accumulation of intracellular glycerol in the absence of high external osmolarity, followed by cell bursting (44, 48). We studied the interaction between the protein and the fungicide fludioxonil by STD-NMR. Discussions with the Dept. of Structural Biology (Prof. Dr. Christiane Ritter) had revealed that this would be the most suitable method because of the size of the protein (121 kDa). The intensities of the characteristic peaks of the aromatic protons of fludioxonil decreased proportionally to the increase of the purified CaNik1p concentration. This can only be explained by a direct interaction between the compound and the protein. Thus, for the first time, CaNik1p, as a member of group III HKs, has been experimentally confirmed as being a direct target for fludioxonil, as an example for antifungals activating the Hog1 MAPK module.

5.4 Effect of mutations and incubation with fludioxonil on the *in vitro* kinase activity of the purified CaNik1p

We analyzed the kinase activity of CaNik1p for two reasons. First, to establish experimentally that the conserved phosphorylatable residues of HisKA (His510) and REC (Asp924) domains are essential for the phosphorylation of CaNik1p and further to correlate such results with data obtained from the fungicidal susceptibility of the transformants that express the corresponding mutated variants of CaNik1p. Second, we wanted to investigate the way that fludioxonil affected the kinase activity of CaNik1p, particularly after observing the direct interaction of the fungicide with purified CaNik1p via STD-NMR. However, biochemical analysis of phosphorelay signaling is more problematic than that of conventional protein kinase signaling systems, because the phosphorylated histidine and aspartate residues are unstable (92). As a consequence, no progress has been made in the production of anti-phosphohistidine or anti-phosphoaspartate antibodies that can be used in Western blots for the detection of proteins phosphorylated at these residues (92). Therefore, we decided to analyze the kinase activity of the purified CaNik1p and its mutated variants after heterologous expression through *in vitro* methods, either by using the Kinase-Glo plus kit or via incubation with radiolabeled [γ-^{32}P] ATP.

Indeed, the *in vitro* kinase activity of group III kinase DhNik1p from *Debaryomyces hansenii* has previously been investigated by using Kinase-Glo plus kit (93). However, no confirmation has been experimentally obtained, for any of the fungal group III HKs, that the conserved phosphorylatable residues of the HisKA (His residue) and the REC (Asp residue) domains are really the only residues that can be phosphorylated in the protein.

ATP concentrations are determined with the Kinase-Glo plus kit. As ATP concentrations decrease with increasing kinase activity, the resulting data are correlated to the enzyme activity. However, they can be falsified by any other ATP-consuming reaction, i.e., ATPase activity. Therefore, we have also used radioactively labeled ATP as a substrate, so that the radioactive phosphate group is incorporated into the protein, and the formation of specific reaction products can be observed. As group III HKs dimerize and autophosphorylate, no additional protein substrate is required.

Results obtained from both the Kinase-Glo plus kit or after incubation with the radiolabeled [γ-^{32}P] ATP were in agreement. The kinase activity of the purified CaNik1p was successfully detected by both methods. Unexpectedly, the protein mutated at His510 still

possessed kinase activity. Even the CaNik1p(H510Q, D924N) double mutant with the additional substitution of the other possible phosphorylatable residue (Asp924) was still able to undergo autophosphorylation as indicated after incubation with the radiolabeled [γ-^{32}P] ATP. Nevertheless, these data do not eliminate the possibility that they are not phosphoaccepting residues in the protein, since additional residues might contribute to the kinase activity of the protein and have not been identified by former researchers.

Surprisingly, kinase activity can still be detected for the proteins CaNik1p(N627D), detected by both methods, and CaNik1p(G633A, G665A), after incubation with the radiolabeled [γ-^{32}P] ATP. However, the presence of the Hsp71 protein as dominant interacting protein with any of the mutants of the HATPase_c domain might explain some of these results. The ATPase activity of Hsp71 (94) could be the reason that the consumption of ATP is tremendously increased when the *in vitro* kinase activity of CaNik1p(N627D) is detected via the Kinase-Glo plus kit. Nonetheless, the ability of the HATPase_c-mutated variants of CaNik1p to undergo autophosphorylation after incubation with the radiolabeled [γ-^{32}P] ATP is still unclear. We cannot account for it by the partial inhibition of the ATP-binding domain, since the autophosphorylation of CaNik1p(N627D) is obviously enhanced in comparison with the wild-type CaNik1p after incubation with the radiolabeled [γ-^{32}P] ATP.

The deletion of HAMP domains was also associated with the enhancement of kinase activity of CaNik1pΔHAMP, by more than two-fold in comparison with CaNik1p, as indicated by data obtained by the Kinase-Glo plus kit. An explanation for this result is a possible HAMP domain–mediated negative regulation of the kinase activity of CaNik1p. In agreement with the results of the full-length protein, additional point mutation of the conserved phosphorylatable residue in the HisKA domain (CaNik1pΔHAMP(H510Q)) does not affect the autophosphorylation of CaNik1pΔHAMP as shown by the results after incubation with the radiolabeled [γ-^{32}P] ATP.

The direct interaction between fludioxonil and purified CaNik1p, as revealed by STD-NMR, stimulated our interest to investigate the way that the fungicide affected the *in vitro* kinase activity of the purified protein. Therefore, the *in vitro* kinase activity of purified CaNik1p was investigated after incubation with fludioxonil, either via the Kinase-Glo plus kit or via incubation with radiolabeled [γ-^{32}P] ATP. Both experiments showed that the fungicide does not interfere with kinase activity. At the beginning of our work, the concept of the inhibition of the HK activity of CaNik1p by the antifungals was strongly accepted by us as an explanation for the growth inhibition observed after fungicidal treatment of *S. cerevisiae*

transformed with *CaNIK1*. Later on, this assumption seemed to be incorrect, as the transformant H510 with *CaNIK1*(H510Q), which is supposed to be unable to exhibit HK activity, is able to grow normally in SG-ura. However, the possibility that the *in vivo* interaction of fludioxonil with CaNik1p might require additional players to be able to affect the HK activity of the protein cannot be neglected.

5.5 Phosphorylation of the Ser1071 residue of CaNik1p

Although the detection of the phosphorylatable serine residue in a HK of TCST seems to be strange, previous researchers have reported similar results with other HKs, e.g., DegS in *B. subtilis* (95) and the amoebal HK DokA (96). These data might explain why CaNik1p(H510Q) still possesses kinase activity, even after the simultaneous substitution of the other possible phosphorylatable residue (Asp924).

5.6 Deletion of HAMP domains in the heterologously expressed CaNik1p

We showed previously that the heterologous expression of truncated variants of the CaNik1p protein, in which several HAMP domains were deleted, did not result in the inhibition of growth of the respective *S. cerevisiae* transformants (47). Whereas in the previous report, only selected HAMP domains were deleted, in the present work, we deleted all HAMP domains from CaNik1p (CaNik1pΔHAMP and CaNik1pΔHAMPup) and observed that the expression of this truncated protein in the transformed *S. cerevisiae* strain was associated with severe growth inhibition. This phenotype could be reversed by additional point mutation in the conserved phosphorylation site of the HisKA domain (H510) or by the expression of *CaNIK1*ΔHAMP (or *CaNIK1*ΔHAMPup) in single gene deletion mutants of the response regulator *SSK1* or in one of the components of the Hog1 MAPK module, namely the MAP2K *PBS2* and the MAPK *HOG1*. This establishes that the inhibition of growth of the transformant upon expression of CaNik1pΔHAMP is dependent on the conserved phosphate-accepting histidine residue of the HisKA domain and the functionality of the Ssk1 – Pbs2 - Hog1 branch of the HOG pathway. Expression of both CaNik1pΔHAMP and CaNik1pΔHAMPup was associated with the same phenotypes in the different transformants of *S. cerevisiae*. Therefore, we focused on the transformant harboring CaNik1pΔHAMP in the following experiments. We could further show that the inhibited growth of the strain ΔHa, expressing CaNik1pΔHAMP, correlated with the constitutive phosphorylation of Hog1p without any external stimulation. This was also abolished after mutation of the conserved phosphate-accepting histidine residue (*CaNIK1*ΔHAMP(H510Q)).

5.7 Both antifungals and HAMP domains activated the Hog1 MAPK module via the same mechanism

Our results allow the conclusion that similarity exists in the mechanism of growth inhibition observed in *S. cerevisiae* transformed with *CaNIK1*, either by fungicidal activity or after deletion of the HAMP domains. First, the conserved phosphate-accepting histidine residue of the HisKA domain (H510) is essential for both treatment with fungicides and the deletion of all the HAMP domains to exert their inhibitory effects on the growth of the yeast. Second, phosphorylation of the MAPK Hog1p is induced after fungicidal treatment or deletion of HAMP domains from *CaNIK1* used to transform *S. cerevisiae.* Moreover, the phosphorylation of Hog1p is totally abolished in both cases after additional substitution of the conserved phosphorylatable His residue of the HisKA domain (H510Q). Third, the components of the Hog1 MAPK module (Pbs2p and Hog1p) in addition to the RR Ssk1p are essential players for the CaNik1pΔHAMP-mediated growth inhibition in the transformed *S. cerevisiae*. Likewise, we have reported that the same players are found to be essential for maintaining the sensitivity of the transformed *S. cerevisiae* to antifungals (47). Based on the previous data, we propose the following model (Fig. 5.1) to explain the similarity between antifungal treatments and the expression of CaNik1pΔHAMP in terms of the activation of the Hog1 MAPK module. Since the only possible mechanism for the activation of the Hog1 MAPK module, i.e., the phosphorylation of the MAPK Hog1p via the RR Ssk1p, is the existence of such RR in the dephosphorylated form (97), we conclude that either the expression of CaNik1pΔHAMP or the fungicidal treatment of *S. cerevisiae* transformed with *CaNIK1* inhibits phosphate transfer to Ssk1p from the active HK ScSln1p via Ypd1p, and that such inhibition requires a phosphorylated CaNik1p at the H510 residue. However, the mechanism by which the phosphorylated CaNik1p interferes with the phosphate transfer in the cascade Sln1p-Ypd1p-Ssk1p needs to be further investigated.

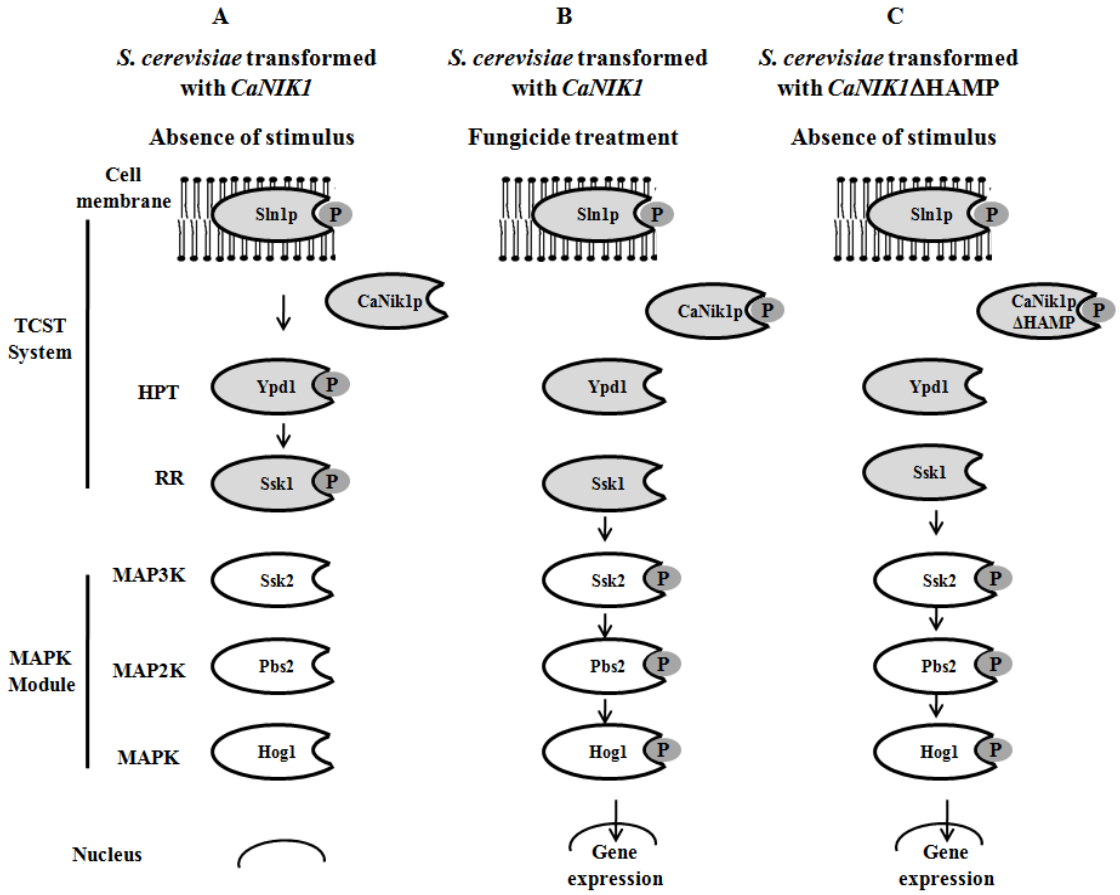


Fig. 5.1: Schematic model for activation of the Hog1 MAPK module after fungicidal treatment or deletion of HAMP domains in *S. cerevisiae* transformed with *CaNIK1*. The scheme shows *S. cerevisiae* transformed with either *CaNIK1* in the absence of stimulus (A) and after treatment with antifungals (B) or transformed with *CaNIK1*ΔHAMP (C).

5.8 Deletion and reintegration of *CaNIK1* in Sc5314

We were interested to delete the *CaNIK1* gene in the wild-type strain Sc5314 for two reasons. First, most of the available information about the relevance of *CaNIK1* in *C. albicans* was obtained from an analysis of its homozygous deletion mutant, which was constructed by the URA blaster method. Because of the disadvantages of this method, as mentioned in 1.4, we wished to reconsider the role of *CaNIK1* in the phenotypic behavior of *C. albicans* via the construction of a homozygous mutant of the gene independently of the utilization of the auxotrophic marker *URA3*. Second, the main reason was to introduce indirectly the mutated variants of *CaNIK1*, which are harbored by the plasmids used for the transformation of *S. cerevisiae*. We hoped that this could be achieved by PCR amplification of the *CaNIK1*-TAG and its mutated variants and the reintegration of these amplicons into the constructed homozygous mutant of *CaNIK1*. The resulting transformants should allow the investigation of

the role of each domain on the phenotypic behavior of the resulting transformants, e.g., hyphal formation on various solid media. Moreover, we were curious to examine the way that deletion of the HAMP domains would affect the growth of resulting transformants of *C. albicans*. The *SAT1* flipper cassette, containing the NST resistance marker, was used for the construction of the *CaNIK1*-deletion and -reintegration cassettes. The homozygous mutant NIKhom2 (*Δnik1*/*Δnik1)* was successfully constructed and confirmed by PCR for the deletion of both alleles of *CaNIK1* and undergoing the flip excision. We decided to delay further confirmation by the complex technique of Southern blotting until the complete design of the *CaNIK1* integration cassettes, as the reintegration of mutated variants of *CaNIK1* was our main aim. However, all the trials to ligate the amplicons of the *CaNIK1*-TAG and its mutated variants with pNIK, amplified by inverse PCR, via the In-fusion enzyme were unsuccessful. To check for the possible absence of nucleotides at the termini of the various DNA fragments (a possibility that would hinder the ligation process), the ends of the different amplicons were examined for their integrity by sequencing, and no missing nucleotides were detected. An acceptable reason for the inability of the In-fusion enzyme to ligate the DNA fragments could be the large size of the resulting plasmid, as the ligation of *CaNIK1*-TAG (3.3 kbp) and the inverse PCR-amplified pNIK (8.4 kbp) would result in a plasmid of 11.7 kbp.

5.9 Conclusions

The present work allowed us to conclude the following:

1- Functional HisKA, HATPase_c, and REC domains are essential for the antifungal mechanism of the compounds activating the Hog1 MAPK module.
2- CaNik1p is a direct target for fludioxonil.
3- Fludioxonil does not interfere with the *in vitro* kinase activity of the protein.
4- Another phosphorylatable residue (Ser1071) exists in CaNik1p beside those that are well known from the structural homology (His510 and Asp924) of HisKA and REC domains, respectively.
5- HAMP domains negatively regulate the activation of the Hog1 MAPK module by CaNik1p.
6- In *S. cerevisiae* transformed with *CaNIK1*, the tested antifungals and deletion of HAMP domains lead to growth inhibition by activation of the Hog1 MAPK module via the response regulator Ssk1p. In both cases, the activation of the Hog1 MAPK module is dependent on the conserved phosphorylation site of the HisKA domain (H510).

6 Outlook

Group III histidine kinases are present in various fungi, which are pathogenic for humans, animals or plants. However, besides the relevance for the susceptibility of these fungi to several classes of fungicides, knowledge about the function of these enzymes is still limited. Thus, neither the regulatory mechanisms of the downstream signal transduction pathways were unambiguously identified nor the endogenous signal leading to activation or inhibition.

As we are now able to produce significant amounts of the protein and mutated variants recombinantly, further studies are possible with the isolated, purified protein.

We observed that the protein was phosphorylated, even when conserved phosphorylatable amino acids were replaced by others. Thus further studies on possible phosphorylatable residues are required. To avoid excessive construction of mutants, a high-throughput method could be used, e.g. a short peptide array, covering all amino acids of the CaNik1p, could be used as substrate in the kinase reaction. Subsequently, the relevance of the detected phosphorylated residues has to be confirmed by mutational analysis.

Moreover, the analysis of the interaction between the fungicides and the protein could be extended to the mutated variants and to other classes of fungicides. The NMR – studies have to be complemented by other methods of structural analysis and by kinetic studies of the interaction.

In addition the interaction between group III histidine kinases and other phosphorelay systems is not well understood. The *S. cerevisiae* transformants are ideal tools to address this question, as the interaction to the endogenous SLN1-YPD1-SSK1 phosphorelay of *S. cerevisiae* could by investigated by examining the possibility of the interaction of CaNik1p with ScSln1p, Ypd1p, or Ssk1p via the yeast two-hybrid system. Previous reports had shown that CaNik1p is not able to complement a deletion of ScSln1p. However, these results should be reevaluated as the unusual codon usage of *C. albicans* was not taken into consideration at that time.

Finally, the function of the different domains of CaNik1p, particularly the HAMP domains, and of the phosphorylation state of the protein for the role of this protein in *Candida albicans* needs to be investigated requiring new efforts to replace the wild-type gene by mutated gene variants in *C. albicans*. Subsequently these investigations could be extended to other pathogenic fungi.

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Appendix I (Equipments and Materials)

Equipment	Type and producing Company
Analysis balance	PC4400, Mettler Sartorius
Automatic micropipettes	Discovery Comfort 2–20, 20–200 and 100-1000 µl, Abimed HTL. Eppendorf Reference 0.5–10, Eppendorf.
Blotting apparatus	Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad
Bunsen Burner	Fireboy, Integra Biosciences Fireboy eco, Integra Biosciences
Centrifuges	5804R, Eppendorf 5402, Eppendorf 5415, Eppendorf
Clean bench	HLB 2448 Heraeus Hera Safe, Therma Electron Corp.
Densitometer	GS-800, Bio-rad
Electroporator	Micropulser, Biorad
Gel documentation system	RH-2, dark room hood, Herolab
Hemocytometer cell counting chamber	Neubauer improved, Assistent Germany
Horizontal gel electrophoresis	HORIZON™ 58 Gel Casting System, Life Technologies
Horizontal gel electrophoresis	Wide Mini-Sub Cell GT Cell, Biorad
Magnetic stirrer plate	MR 2002, Heidolph
Microplate shaker incubator	Titramax 1000, Heidolph
Microplate spectrophotometer	µQuant, BioTek
Microplate reader for chemiluminescence	Synergy™4, BioTek
Microscope	Zeiss, Germany
Microwave	Hi Speed, Brother
Mikro-Dismembrator	Mikro-Dismembrator U, B. Braun Biotech International
Multichannel pipette	Transferpette 30–300 µl, Brand
NanoDrop 1000 spectrophotometer, Thermo Scientific.	Determination of DNA and purified CaNik1p concentrations
Oven	UN, Memmert
pH meter	pH 211 Microprocessor pH Meter, Hanna Instruments
Phosphorimage analyzer	BAS2500, Fujifilm
Pipet Aid	Easypet, Eppendorf CellMate Matrix, Thermo Scientific
Pipette Dispenser	Eppendorf
Polyacrylamide Gel Electrophoresis chamber	Minigel-Twin, Biometra
Power supply	Powerpac 1000 and 200 , Biorad
Rocking shaker	Duomax 1030, Heidolph
Scanning microplate spectrophotometer	µQuant, Biotek

Shaker incubator	Multitron Standard, Infors HT
Thermocycler	Personal Cycler, Biometra
Thermomixers	5437, Eppendorf Comfort 5355R, Eppendorf Thermostat 5320, Eppendorf
Vaccum pump	XF54 230 50, Millipore KN4, Neuberger
Vortex	Vortex Genie 2, Scientific Industries
Waterbath	Wbn 14, Memmert
Western blot imaging system	LAS-3000, Fujifilm
Material and company	**Used in**
Minisart Membranfilter (pore size 0.2 µm),	Sterile filtration of volumes less than 50 ml
Bottle-Top 500 ml Filter 0.2 µm, Nalgene 595-4520	Sterile filtration of volumes more than 50 ml
Steriflip-NY Filter 0.2 µm, Millipore.	- Sterile filtration of volumes less than 50 ml. - In CaNik1p purification, for separation of the Ni^{+2} agarose beads from the eluate solution of the protein.
Pipette tips Starlab S1111-0006, S1111-2021 and S1111-3000	For pipetting with the automatic micropipettes
Combitipsplus 5 and 10 ml, Eppendorf	Repeated transfer of the same volume by using the pipette dispenser
Safe-Lock-Tubes (2ml), Eppendorf	Centrifugation of volumes lower that 2 ml
Cryovials 2ml, Corning 5380542	Storage of aliquots of cultures as stocks
Centrifuge tubes 15 ml und 50 ml, Corning 430829, 430791 und 430897	Centrifugation of volumes more than 2 ml
PCR tubes 0,2 ml, Brand 781300	In PCR
Reservoirs, 60 ml solution volume with cover, Eppendorf	To allow the use of the multichannel pipettes in determination of protein concentration by using BCA method and for the *in vitro* kinase assay of the CaNik1p.
Disposable Petri dishes	For cultivation of microorganism on solid media
Immunoblot PVDF Membrane, Bio-Rad 162-077	Western blot
Extra thick filter paper, Bio-Rad 1703960	Western blot
96-well Costar microtiter plates with white opaque-bottom, Corning 3917	Measurement of luminescence in the kinase assay by using Kinase Glo plus Kit.
CytoOne 96-Well Plate, TC-treated, USA Scientific.	Cultivation of transformed yeast strains in the determination their susceptibility to antifungals
96-well microtiter plates without cover, W.O. Schmidt	For measurement of OD of cultures and in protein determination
30, 50, and 100 kDa MWCO Vivaspin20 and 500 spin columns, Sartorius	CaNik1p purification

Appendix II (Mascot search results and peptide mass fingerprint after MALD-TOF analysis of certain proteins)

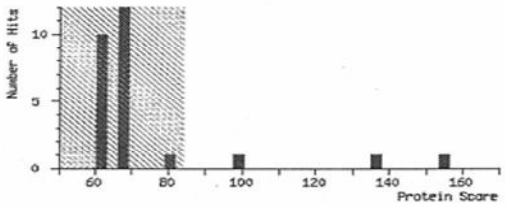
Purified CaNik1p

{MATRIX} {SCIENCE} **Mascot Search Results**

```
User            :
Email           : ame@helmholtz-hzi.de
Search title    :
Database        : NCBInr 20110131 (12852469 sequences; 4389733743 residues)
Timestamp       : 8 Feb 2011 at 10:42:23 GMT
Top Score       : 155 for gi|3243089, histidine kinase [Candida albicans]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 84 are significant (p<0.05).



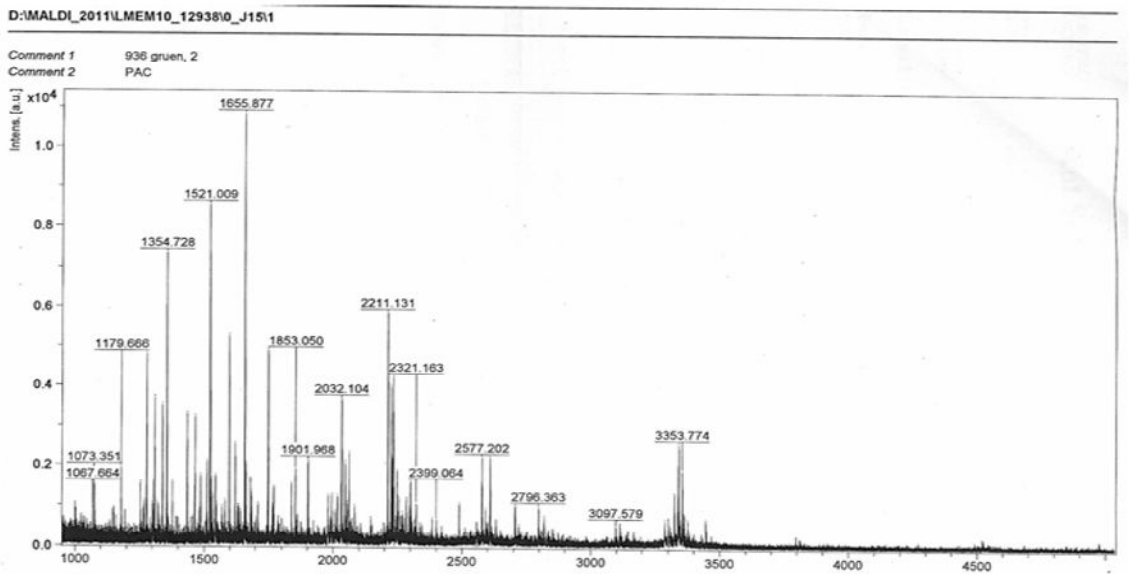
Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits 10

Re-Search All | Search Unmatched

```
1.    gi|3243089     Mass: 119444    Score: 155    Expect: 4.1e-09  Matches: 33
      histidine kinase [Candida albicans]
      gi|3850150     Mass: 119371    Score: 155    Expect: 4.1e-09  Matches: 33
      histidine kinase [Candida albicans]
      gi|2911164     Mass: 119344    Score: 144    Expect: 5.1e-08  Matches: 32
      CaNIK1 [Candida albicans]
```



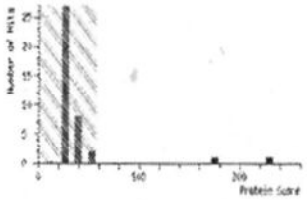
Interacting protein with CaNik1p(N627D) and CaNik1p(G663A, G665A)

MATRIX SCIENCE **Mascot Search Results**

```
User          :
Email         : ame@helmholtz-hzi.de
Search title  : 620white
Database      : SwissProt 2012_09 (538010 sequences; 190998508 residues)
Taxonomy      : Fungi (30775 sequences)
Timestamp     : 16 Oct 2012 at 07:54:22 GMT
Top Score     : 229 for HSP71_YEAST, Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 57 are significant (p<0.05).



Concise Protein Summary Report

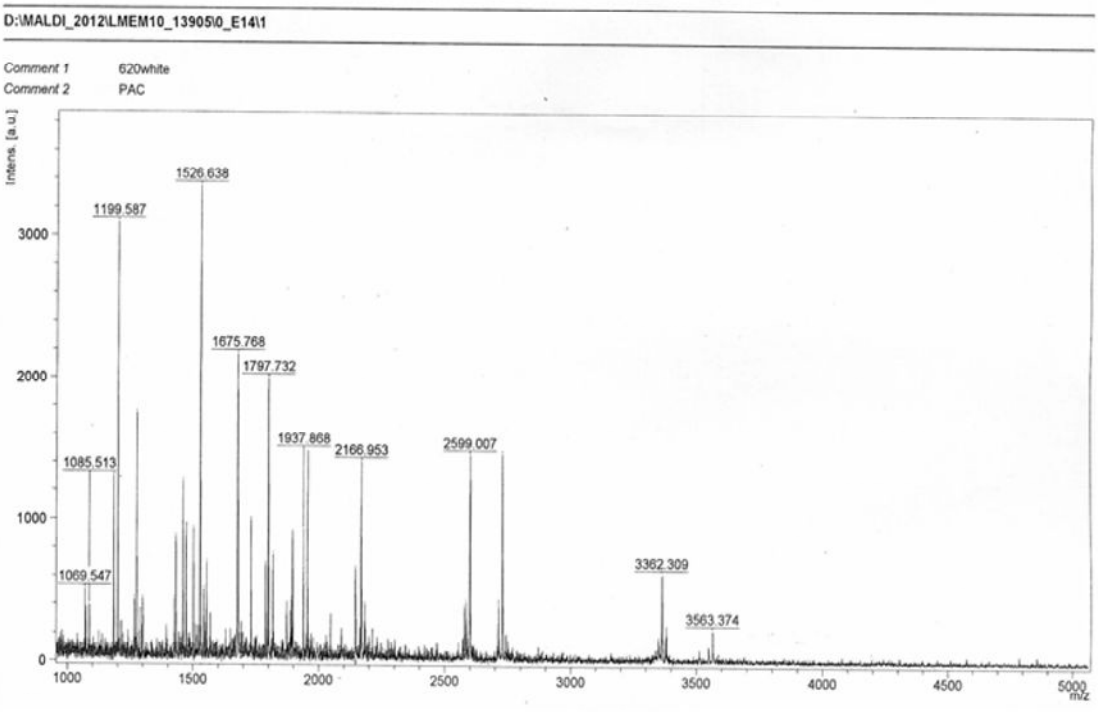
Format As: Concise Protein Summary — Help

Significance threshold p< 0.05 Max. number of hits 10

Preferred taxonomy: All entries

Re-Search All | Search Unmatched

1. HSP71_YEAST **Mass:** 69786 **Score:** 229 **Expect:** 3.9e-19 **Matches:** 20
   Heat shock protein SSA1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4



Appendix III (Sequences of various mutated variants of *CaNIK1*)

Sequence of pYES2-*CaNIK1*-TAG, *CaNIK1* starts from 5794 (ATG) till 9105 (TGA):

```
   1 tcgagtcgac ttgattctag agggccgcat catgtaatta gttatgtcac
  51 gcttacattc acgccctccc cccacatccg ctctaaccga aaaggaagga
 101 gttagacaac ctgaagtcta ggtccctatt tatttttta tagttatgtt
 151 agtattaaga acgttattta tatttcaaat ttttcttttt tttctgtaca
 201 gacgcgtgta cgcatgtaac attatactga aaaccttgct tgagaaggtt
 251 ttgggacgct cgaaggcttt aatttgcggc cctgcattaa tgaatcggcc
 301 aacgcgcggg gagaggcggt ttgcgtattg ggcgctcttc cgcttcctcg
 351 ctcactgact cgctgcgctc ggtcgttcgg ctgcggcgag cggtatcagc
 401 tcactcaaag gcggtaatac ggttatccac agaatcaggg gataacgcag
 451 gaaagaacat gtgagcaaaa ggccagcaaa agcccaggaa ccgtaaaaag
 501 gccgcgttgc tggcgttttt ccataggctc cgccccccctg acgagcatca
 551 caaaaatcga cgctcaagtc agaggtggcg aaacccgaca ggactataaa
 601 gataccaggc gtttcccccct ggaagctccc tcgtgcgctc tcctgttccg
 651 accctgccgc ttaccggata cctgtccgcc tttctccctt cgggaagcgt
 701 ggcgctttct catagctcac gctgtaggta tctcagttcg gtgtaggtcg
 751 ttcgctccaa gctgggctgt gtgcacgaac cccccgttca gcccgaccgc
 801 tgcgccttat ccggtaacta tcgtcttgag tccaacccgg taagacacga
 851 cttatcgcca ctggcagcag ccactggtaa caggattagc agagcgaggt
 901 atgtaggcgg tgctacagag ttcttgaagt ggtggcctaa ctacggctac
 951 actagaagga cagtatttgg tatctgcgct ctgctgaagc cagttacctt
1001 cggaaaaaga gttggtagct cttgatccgg caaacaaacc accgctggta
1051 gcggtggttt ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga
1101 tctcaagaag atcctttgat cttttctacg gggtctgacg ctcagtggaa
1151 cgaaaactca cgttaaggga ttttggtcat gagattatca aaaaggatct
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1251 atatatgagt aaacttggtc tgacagttac caatgcttaa tcagtgaggc
1301 acctatctca gcgatctgtc tatttcgttc atccatagtt gcctgactcc
1351 ccgtcgtgta gataactacg atacgggagc gcttaccatc tggccccagt
1401 gctgcaatga taccgcgaga cccacgctca ccggctccag atttatcagc
1451 aataaaccag ccagccggaa gggccgagcg cagaagtggt cctgcaactt
1501 tatccgcctc cattcagtct attaattgtt gccgggaagc tagagtaagt
1551 agttcgccag ttaatagttt gcgcaacgtt gttggcattg ctacaggcat
1601 cgtggtgtca ctctcgtcgt ttggtatggc ttcattcagc tccggttccc
1651 aacgatcaag gcgagttaca tgatccccca tgttgtgcaa aaaagcggtt
1701 agctccttcg gtcctccgat cgttgtcaga agtaagttgg ccgcagtgtt
1751 atcactcatg gttatggcag cactgcataa ttctcttact gtcatgccat
1801 ccgtaagatg cttttctgtg actggtgagt actcaaccaa gtcattctga
1851 gaatagtgta tgcggcgacc gagttgctct tgcccggcgt caatacggga
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1951 gttcttcggg gcgaaaactc tcaaggatct taccgctgtt gagatccagt
2001 tcgatgtaac ccactcgtgc acccaactga tcttcagcat cttttacttt
2051 caccagcgtt tctgggtgag caaaaacagg aaggcaaaat gccgcaaaaa
2101 agggaataag ggcgacacgg aaatgttgaa tactcatact cttcctttt
2151 caatgggtaa taactgatat aattaaattg aagctctaat ttgtgagttt
2201 agtatacatg catttactta taatacagtt ttttagtttt gctggccgca
2251 tcttctcaaa tatgcttccc agcctgcttt tctgtaacgt tcaccctcta
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Sequence of *CaNIK1*(H510Q) in pYES2-*CaNIK1*(H510Q):

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Sequence of *CaNIK1*(D924N) in pYES2-*CaNIK1*(D924N):

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Sequence of *CaNIK1*(H510Q, D924N) in pYES2-*CaNIK1*(H510Q, D924N):

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Sequence of *CaNIK1*(N627D) in pYES2-*CaNIK1*(N627D):

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Sequence of *CaNIK1*(G663A, G665A) in pYES2-*CaNIK1*(G663A, G665A):

atgaaccccactaaaaaaccacggttatcaccaatgcagccctctgtttttgaaatactc
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Sequence of pYES2-*CaNIK1*ΔHAMPup (7629 bp), *CaNIK1*ΔHAMPup starts from 507 (ATG) till 2364 (TGA):

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   1 ACGGATTAGA AGCCGCCGAG CGGGTGACAG CCCTCCGAAG GAAGACTCTC
  51 CTCCGTGCGT CCTCGTCTTC ACCGGTCGCG TTCCTGAAAC GCAGATGTGC
 101 CTCGCGCCGC ACTGCTCCGA ACAATAAAGA TTCTACAATA CTAGCTTTTA
 151 TGGTTATGAA GAGGAAAAAT TGGCAGTAAC CTGGCCCCAC AAACCTTCAA
 201 ATGAACGAAT CAAATTAACA ACCATAGGAT GATAATGCGA TTAGTTTTTT
 251 AGCCTTATTT CTGGGGTAAT TAATCAGCGA AGCGATGATT TTTGATCTAT
 301 TAACAGATAT ATAAATGCAA AAACTGCATA ACCACTTTAA CTAATACTTT
 351 CAACATTTTC GGTTTGTATT ACTTCTTATT CAAATGTAAT AAAAGTATCA
 401 ACAAAAAATT GTTAATATAC CTCTATACTT TAACGTCAAG GAGAAAAAAC
 451 CCCGGATCGG ACTACTAGCA GCTGTAATAC GACTCACTAT AGGGAATATT
 501 AAGCTTatga ggaatactgc ggctagagaa gctgctgagc ttgccaatag
 551 tgcgaaatcc gagtttctag cgaacatgtc gcatgagata cgtacaccat
 601 tgaatgggat tattggtatg actcagttgt cgcttgatac agagttgaca
 651 cagtaccaac gagagatgtt gtcgattgtg cataacttgg caaattcctt
 701 gttgaccatt atagacgata tattggatat ttctaagatt gaggcgaata
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 851 ctatcaatgt gattcatcgt ttccagataa tcttattgga gatagtttta
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1051 tagagaaaga caaattggga ttgattttcg ataccttctg tcaagctgat
1101 ggttctacta caagaaagtt tggtggtaca ggtttagggt tgtcaatttc
1151 caaacagttg atacatttaa tgggtggaga gatatgggtt acttcggagt
1201 atggatcagg gtcaaacttt tattttacgg tgtgcgtgtc gccatctaat
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1501 ttccgttggt tttggtccat cattctattc cacagttgaa tatgagagta
1551 tgtattgatt tggggatttc ttcctatgca aatacgccat gttcgatcac
1601 ggacttggca agtgcgatta taccagcgtt ggagtcgaga tccatatcac
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1651 agaactcaga cgagtcggtg aggtacaaaa tattactagc agaggacaac
1701 ctcgtcaatc agaaacttgc agttaggata ttagaaaagc aagggcattc
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1951 tgttaggtga tagagaaaag tcattggcaa aggggatgga cgattatgtg
2001 agtaagccat tgaagccgaa attgttaatg cagacgataa acaagtgtat
2051 tcataatatt aaccagttga aagaattgtc gagaaatagt aggggtagcg
2101 attttgcaaa gaagatgacc cgaaacacac ccggaagcac gacccgtcag
2151 gggagtgatg aggggagtgt agaggacatg attggggaca ctccccgtca
2201 agggagtgtt gagggagggg gtacaagtag cagaccagta cagagaaggt
2251 ctgccacaga ggggtcgatc actacaatta gtgaacaaat cgaccggtta
2301 cgtaaggatc cgggtgatta taaagatgac gacgataaag gaggacatca
2351 tcaccatcac cactgacTCT AGAGGGCCGC ATCATGTAAT TAGTTATGTC
2401 ACGCTTACAT TCACGCCCTC CCCCCACATC CGCTCTAACC GAAAAGGAAG
2451 GAGTTAGACA ACCTGAAGTC TAGGTCCCTA TTTATTTTTT TATAGTTATG
2501 TTAGTATTAA GAACGTTATT TATATTTCAA ATTTTTCTTT TTTTTCTGTA
2551 CAGACGCGTG TACGCATGTA ACATTATACT GAAAACCTTG CTTGAGAAGG
2601 TTTTGGGACG CTCGAAGGCT TTAATTTGCG GCCCTGCATT AATGAATCGG
2651 CCAACGCGCG GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT TCCGCTTCCT
2701 CGCTCACTGA CTCGCTGCGC TCGGTCGTTC GGCTGCGGCG AGCGGTATCA
2751 GCTCACTCAA AGGCGGTAAT ACGGTTATCC ACAGAATCAG GGGATAACGC
2801 AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGCCCAGG AACCGTAAAA
2851 AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC TGACGAGCAT
2901 CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA CAGGACTATA
2951 AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC
3001 CGACCCTGCC GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC
3051 GTGGCGCTTT CTCATAGCTC ACGCTGTAGG TATCTCAGTT CGGTGTAGGT
3101 CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT CAGCCCGACC
3151 GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC GGTAAGACAC
3201 GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA GCAGAGCGAG
3251 GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT
3301 ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC
3351 TTCGGAAAAA GAGTTGGTAG CTCTTGATCC GGCAAACAAA CCACCGCTGG
3401 TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC AGAAAAAAAG
3451 GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA CGCTCAGTGG
3501 AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT CAAAAAGGAT
3551 CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA

3601 GTATATATGA GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG
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3701 CCCCGTCGTG TAGATAACTA CGATACGGGA GCGCTTACCA TCTGGCCCCA
3751 GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA
3801 GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC
3851 TTTATCCGCC TCCATTCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA
3901 GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGGCAT TGCTACAGGC
3951 ATCGTGGTGT CACTCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC
4001 CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG
4051 TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG
4101 TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA CTGTCATGCC
4151 ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT
4201 GAGAATAGTG TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAATACGG
4251 GATAATAGTG TATCACATAG CAGAACTTTA AAAGTGCTCA TCATTGGAAA
4301 ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA
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5101 GTCAGCAAAT TTTCTGTCTT CGAAGAGTAA AAAATTGTAC TTGGCGGATA
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5351 TGAGTAGCAG CACGTTCCTT ATATGTAGCT TTCGACATGA TTTATCTTCG
5401 TTTCCTGCAG GTTTTTGTTC TGTGCAGTTG GGTTAAGAAT ACTGGGCAAT
5451 TTCATGTTTC TTCAACACTA CATATGCGTA TATATACCAA TCTAAGTCTG

5501 TGCTCCTTCC TTCGTTCTTC CTTCTGTTCG GAGATTACCG AATCAAAAAA
5551 ATTTCAAAGA AACCGAAATC AAAAAAAAGA ATAAAAAAAA AATGATGAAT
5601 TGAATTGAAA AGCTAGCTTA TCGATGATAA GCTGTCAAAG ATGAGAATTA
5651 ATTCCACGGA CTATAGACTA TACTAGATAC TCCGTCTACT GTACGATACA
5701 CTTCCGCTCA GGTCCTTGTC CTTTAACGAG GCCTTACCAC TCTTTTGTTA
5751 CTCTATTGAT CCAGCTCAGC AAAGGCAGTG TGATCTAAGA TTCTATCTTC
5801 GCGATGTAGT AAAACTAGCT AGACCGAGAA AGAGACTAGA AATGCAAAAG
5851 GCACTTCTAC AATGGCTGCC ATCATTATTA TCCGATGTGA CGCTGCAGCT
5901 TCTCAATGAT ATTCGAATAC GCTTTGAGGA GATACAGCCT AATATCCGAC
5951 AAACTGTTTT ACAGATTTAC GATCGTACTT GTTACCCATC ATTGAATTTT
6001 GAACATCCGA ACCTGGGAGT TTTCCCTGAA ACAGATAGTA TATTTGAACC
6051 TGTATAATAA TATATAGTCT AGCGCTTTAC GGAAGACAAT GTATGTATTT
6101 CGGTTCCTGG AGAAACTATT GCATCTATTG CATAGGTAAT CTTGCACGTC
6151 GCATCCCCGG TTCATTTTCT GCGTTTCCAT CTTGCACTTC AATAGCATAT
6201 CTTTGTTAAC GAAGCATCTG TGCTTCATTT TGTAGAACAA AAATGCAACG
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6301 CAGAAATGCA ACGCGAAAGC GCTATTTTAC CAACGAAGAA TCTGTGCTTC
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6451 TATTTTACCA ACAAAGAATC TATACTTCTT TTTTGTTCTA CAAAAATGCA
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6551 CCTTTGTGCG CTCTATAATG CAGTCTCTTG ATAACTTTTT GCACTGTAGG
6601 TCCGTTAAGG TTAGAAGAAG GCTACTTTGG TGTCTATTTT CTCTTCCATA
6651 AAAAAAGCCT GACTCCACTT CCCGCGTTTA CTGATTACTA GCGAAGCTGC
6701 GGGTGCATTT TTTCAAGATA AAGGCATCCC CGATTATATT CTATACCGAT
6751 GTGGATTGCG CATACTTTGT GAACAGAAAG TGATAGCGTT GATGATTCTT
6801 CATTGGTCAG AAAATTATGA ACGGTTTCTT CTATTTTGTC TCTATATACT
6851 ACGTATAGGA AATGTTTACA TTTTCGTATT GTTTTCGATT CACTCTATGA
6901 ATAGTTCTTA CTACAATTTT TTTGTCTAAA GAGTAATACT AGAGATAAAC
6951 ATAAAAAATG TAGAGGTCGA GTTTAGATGC AAGTTCAAGG AGCGAAAGGT
7001 GGATGGGTAG GTTATATAGG GATATAGCAC AGAGATATAT AGCAAAGAGA
7051 TACTTTTGAG CAATGTTTGT GGAAGCGGTA TTCGCAATGG GAAGCTCCAC
7101 CCCGGTTGAT AATCAGAAAA GCCCCAAAAA CAGGAAGATT GTATAAGCAA
7151 ATATTTAAAT TGTAAACGTT AATATTTTGT TAAAATTCGC GTTAAATTTT
7201 TGTTAAATCA GCTCATTTTT TAACGAATAG CCCGAAATCG GCAAAATCCC
7251 TTATAAATCA AAAGAATAGA CCGAGATAGG GTTGAGTGTT GTTCCAGTTT
7301 CCAACAAGAG TCCACTATTA AAGAACGTGG ACTCCAACGT CAAAGGGCGA
7351 AAAAGGGTCT ATCAGGGCGA TGGCCCACTA CGTGAACCAT CACCCTAATC
7401 AAGTTTTTTG GGGTCGAGGT GCCGTAAAGC AGTAAATCGG AAGGGTAAAC

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 7451 GGATGCCCCC ATTTAGAGCT TGACGGGGAA AGCCGGCGAA CGTGGCGAGA
 7501 AAGGAAGGGA AGAAAGCGAA AGGAGCGGGG GCTAGGGCGG TGGGAAGTGT
 7551 AGGGGTCACG CTGGGCGTAA CCACCACACC CGCCGCGCTT AATGGGGCGC
 7601 TACAGGGCGC GTGGGGATGA TCCACTAGT
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Sequence of *CaNIK1*ΔHAMPup(H510Q) in pYES2-*CaNIK1*ΔHAMPup(H510Q):

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Sequence of pYES2-*CaNIK1*ΔHAMP, *CaNIK1*ΔHAMP (7905 bp) starts from 507(ATG) till 2548 (TGA):

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   51 CTCCGTGCGT CCTCGTCTTC ACCGGTCGCG TTCCTGAAAC GCAGATGTGC
  101 CTCGCGCCGC ACTGCTCCGA ACAATAAAGA TTCTACAATA CTAGCTTTTA
  151 TGGTTATGAA GAGGAAAAAT TGGCAGTAAC CTGGCCCCAC AAACCTTCAA
  201 ATGAACGAAT CAAATTAACA ACCATAGGAT GATAATGCGA TTAGTTTTTT
  251 AGCCTTATTT CTGGGGTAAT TAATCAGCGA AGCGATGATT TTTGATCTAT
  301 TAACAGATAT ATAAATGCAA AAACTGCATA ACCACTTTAA CTAATACTTT
  351 CAACATTTTC GGTTTGTATT ACTTCTTATT CAAATGTAAT AAAAGTATCA
  401 ACAAAAAATT GTTAATATAC CTCTATACTT TAACGTCAAG GAGAAAAAAC
  451 CCCGGATCGG ACTACTAGCA GCTGTAATAC GACTCACTAT AGGGAATATT
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 501 AAGCTTatga accccactaa aaaaccacgg ttatcaccaa tgcagccctc
 551 tgtttttgaa atactcaacg accctgagct ttatagtcag cactgtcata
 601 gccttaggga aacacttctt gaccatttca accatcaagc tacacttatc
 651 gacacttatg aacatgaact agaaaaatcc aaaaacgcga acaggaatac
 701 tgcggctaga gaagctgctg agcttgccaa tagtgcgaaa tccgagtttc
 751 tagcgaacat gtcgcatgag atacgtacac cattgaatgg gattattggt
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1051 cgtttccaga taatcttatt ggagatagtt ttagattacg acaagttatt
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1151 tgttagtgtg aaaaagtctg ataaaatggt gttagatagt aagttgttgt
1201 tagaggtttg tgttagcgac acgggaatag gtatagagaa agacaaattg
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1351 taatgggtgg agagatatgg gttacttcgg agtatggatc agggtcaaac
1401 ttttatttta cggtgtgcgt gtcgccatct aatattagat atactcgaca
1451 aaccgaacaa ttattaccat ttagttccca ttatgtgtta tttgtatcga
1501 ctgagcatac tcaagaagaa cttgatgtgt tgagagatgg aattatagaa
1551 cttggattga tacctataat agtgagaaat attgaagatg caacattgac
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1651 aaaagttgag gttgttatcg gaggttaaat atattccgtt ggttttggtc
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2151 aagtcattgg caaaggggat ggacgattat gtgagtaagc cattgaagcc
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2351 tgtagaggac atgattgggg acactccccg tcaagggagt gttgagggag
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2451 atcactacaa ttagtgaaca aatcgaccgg ttacgtaagg atccgggtga
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2851 TATACTGAAA ACCTTGCTTG AGAAGGTTTT GGGACGCTCG AAGGCTTTAA
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2951 GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG
3001 TCGTTCGGCT GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG
3051 TTATCCACAG AATCAGGGGA TAACGCAGGA AAGAACATGT GAGCAAAAGG
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3151 ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG
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3251 AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC
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3351 TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT
3401 GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC
3451 GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC
3501 ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT
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3701 GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT CCTTTGATCT
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4201 GCAACGTTGT TGGCATTGCT ACAGGCATCG TGGTGTCACT CTCGTCGTTT
4251 GGTATGGCTT CATTCAGCTC CGGTTCCCAA CGATCAAGGC GAGTTACATG
4301 ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG

4351 TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA
4401 CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC
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6201 TGAGGAGATA CAGCCTAATA TCCGACAAAC TGTTTTACAG ATTTACGATC

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6251 GTACTTGTTA CCCATCATTG AATTTTGAAC ATCCGAACCT GGGAGTTTTC
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6951 CGTTTACTGA TTACTAGCGA AGCTGCGGGT GCATTTTTTC AAGATAAAGG
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7051 AGAAAGTGAT AGCGTTGATG ATTCTTCATT GGTCAGAAAA TTATGAACGG
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7151 CGTATTGTTT TCGATTCACT CTATGAATAG TTCTTACTAC AATTTTTTTG
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7551 GATAGGGTTG AGTGTTGTTC CAGTTTCCAA CAAGAGTCCA CTATTAAAGA
7601 ACGTGGACTC CAACGTCAAA GGGCGAAAAA GGGTCTATCA GGGCGATGGC
7651 CCACTACGTG AACCATCACC CTAATCAAGT TTTTTGGGGT CGAGGTGCCG
7701 TAAAGCAGTA AATCGGAAGG GTAAACGGAT GCCCCCATTT AGAGCTTGAC
7751 GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA AGCGAAAGGA
7801 GCGGGGGCTA GGGCGGTGGG AAGTGTAGGG GTCACGCTGG GCGTAACCAC
7851 CACACCCGCC GCGCTTAATG GGGCGCTACA GGGCGCGTGG GGATGATCCA
7901 CTAGT
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Sequence of *CaNIK1*ΔHAMP(H510Q) in pYES2-*CaNIK1*ΔHAMP(H510Q):

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